A STUDY OF THE PATHOGENESIS OF LOUPING-ILL

by

Hugh Watt Reid

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SUMMARY

Studies of the pathogenesis of louping-ill virus infection were undertaken to define the disease process in susceptible sheep and were extended to consider the influence of maternally derived antibody on the course of infection. In addition, the possible role of the red grouse in the epidemiology of louping-ill was investigated.

Sheep inoculated subcutaneously with louping-ill virus developed a viraemia within 24 hours, maximum titres being reached two to three days later. In most sheep the virus titres were sufficient to infect the natural vector, Ixodes ricinus. The viraemia subsequently fell rapidly and the fall was associated with the appearance of both serum haemagglutination-inhibiting and neutralizing antibodies. Sheep surviving infection had shorter and lower viraemias and developed antibodies faster than animals that succumbed. There was, therefore, a definite association between the duration and magnitude of the viraemia and the appearance of serum antibody and survival.

The antibody response of sheep to infection with louping-ill virus is typical of the response of other species to infection with togaviruses. The initial serum haemagglutination-inhibiting antibody activity was primarily due to IgM which was progressively replaced by IgG; no IgM was detected after the 22nd day.

The cerebrospinal fluid of sheep inoculated subcutaneously

with louping-ill virus invariably contained haemagglutination-inhibiting antibody. As with serum much of this antibody was IgM during the acute phase of infection, whereas with survivors it was entirely IgG. This pattern was reflected in the total levels of IgM and IgG in cerebrospinal fluid; IgM was present in cerebrospinal fluid during the acute phase, but could not be detected in cerebrospinal fluid from survivors or control animals. The relative proportion of antibody to louping-ill virus detected in cerebrospinal fluid of infected animals was considerably greater than that to a serum marker not involved in the disease process. It is therefore concluded that antibody specific to louping-ill virus is locally produced in the central nervous system.

Serum antibody in lambs derived from colostrum was protective. Infection of lambs with high levels of antibody was completely aborted. Lambs with low levels of haemagglutination-inhibiting antibodies experienced infection in the absence of viraemia, but with the development of an immune response.

Approximately 80 per cent of red grouse infected peripherally with louping-ill virus died and the levels of viraemia achieved were sufficient to infect the vector. As with sheep, the levels of virus that developed in surviving birds were lower than in birds that succumbed. The cessation of viraemia was also associated with the appearance of high titres of serum antibody to louping-ill virus.

The epidemiological implications of these findings are discussed. It is concluded that throughout much of the hill grazing of Scotland where louping-ill is endemic, sheep are the principal vertebrate host; lambs that acquire colostral antibody to louping-ill virus will be protected throughout the period of tick activity in the spring, ewe hoggs in their second spring would appear to be the most important component of the sheep flock in the maintenance of louping-ill virus. Feral species probably play a very minor role.

An historical appraisal indicates that louping-ill virus has been introduced to the grouse moors of Scotland in the comparative recent past due to the introduction of large scale sheep farming during the 19th century. Prior to this grouse probably did not encounter infection. The grouse - louping-ill virus, host-virus relationship has therefore only recently evolved which explains the marked susceptibility of the species to infection with virus.

CONTENTS

INTRODUCTION

The term "louping-ill" has been employed by farmers and shepherds in the Border districts of England and Scotland to describe a clinical condition of sheep (M'Fadzean, 1900), and was in common usage in the 18th century (Duncan, 1807; Cleish-bottam, 1816). It was, however, descriptive, and appears to have covered a variety of conditions including pyaemic spinal meningitis, gastritis, enteritis, thorn-in-the-foot, tapeworm cysts, woolball, pyaemia, pneumonia and fog-fever as well as the disease now defined as louping-ill (M'Fadzean, 1894; M'Gowan and Rettie, 1913). Evidence that a specific clinical condition of sheep involving locomotor dysfunction existed within this general term emerged from work commenced in 1929 at the Moredun Institute.

The clinical course of the naturally occurring and experimentally transmitted disease was described (Poole, Brownlee and Wilson, 1930) and the neurohistopathological lesions associated with the disease were reported (Brownlee and Wilson, 1932). The aetiological agent was established to be a filterable virus (Greig, Brownlee, Wilson and Gordon, 1931) which could be transmitted by Ixodes ricinus. Transstadial but not transovarial transmission in ticks was demonstrated (MacLeod and Gordon, 1932). A vaccine capable of protecting sheep was developed shortly thereafter (Gordon, 1934) and interest in the condition waned. The epidemiology and pathogenesis of louping-ill was, therefore, only studied to a limited extent.

Louping-ill virus is now recognised to be one of eight named members of the closely inter-related tick-borne flaviviruses (Andrews and Pereira, 1972). It is confined to the United Kingdom and is present in Scotland and North England (Greig et al, 1931), Ireland (Walton and Kennedy, 1966) and Wales (Hughes and Kershaw, 1957). There is, however, no detailed information on its distribution within these regions.

Disease associated with infection is most frequently seen in sheep, but natural cases have also been described in cattle (Dunn, 1952), horses (Fletcher and Galloway, 1937), dogs (MacKenzie, Smith and Muir, 1973) and man (Brewis, Neubauer and Hurst, 1949; Williams and Thorburn, 1962). Following experimental infection, encephalitis has been produced in pigs (Dow and McFerran, 1964), monkeys (Hurst, 1931), mice (Alston and Gibson, 1931), hamsters (Grešíková, Albrecht and Ernek, 1961) field voles (Microtus agrestis) (Findlay and Elton, 1933; Seamer and Zlotnik, 1970) and guinea pigs (Zlotnik, Carter and Grant, 1971).

Naturally occurring disease is, however, primarily associated with sheep in which species it is characteristically biphasic. The first phase is associated with fever and viraemia followed several days later in a proportion of infections by inco-ordination, paralysis and death (Poole et al, 1930). Intracerebral inoculation of virus always has a fatal outcome (Gordon, Brownlee, Wilson and MacLeod, 1932a; Edward , 1947). In explanation of the variable clinical outcome following peripheral infection a

number of factors such as age, nutritional status, stress and concurrent disease have been suggested as important in potentiating viral invasion of the central nervous system (MacLeod and Gordon, 1932; Smith, McMahon, O'Reilly, Wilson and Robertson, 1964), but none of these hypotheses have been tested. There was, therefore, a requirement to analyse the dynamics of infection before speculating on what factors might influence the outcome or on the biological significance of infection with respect to transmission to the vector.

The importance of maternally transferred antibody in protecting lambs from infection has been stressed (Wilson and Gordon, 1948; Williams and Thorburn, 1961; Smith et al, 1964; Smith, 1969), but it has not been examined critically in relation to disease in lambs or transmission to I. ricinus.

There was some evidence to suggest that a number of feral species naturally became infected with louping-ill virus. The isolation of virus from a shrew (Sorex araneus) and two wood mice (Apodemus sylvaticus) (Smith, Varma and McMahon, 1964) and three red grouse (Lagopus scoticus) (Williams, Thorburn and Ziffo, 1963; Watt, Brotherston and Campbell, 1963) had been reported and the presence of antibody in red deer (Cervus elaphus) serum had been recorded (Dunn, 1960). The epidemiological significance of these observations remain unknown.

Attempts to elucidate the epidemiology of louping-ill in the absence of an intimate knowledge of the normal course of

infection is not possible. The studies reported here were, therefore, undertaken with the purpose of defining the disease process in susceptible sheep and were extended to consider the influence of maternally derived antibody. Infection of one feral species was also investigated to extend the understanding of the epidemiology of louping-ill virus in the Scottish environment.

CHAPTER I

MATERIALS AND METHODS EMPLOYED: I VIRUS ASSAY.

INTRODUCTION

At the commencement of the study the routine method of virus assay employed in the laboratory at the Moredun Institute was by intracerebral (i.c.) inoculation of mice; a method originally described by Alston and Gibson, (1931). Louping-ill virus induced, specific cytopathopathic effects in tissue culture had been reported (Williams, 1958) and Madrid and Porterfield (1969) described a method of plaquing louping-ill virus. The possibility of adapting tissue culture methods for routine assays was therefore investigated. This chapter describes the virus isolates employed and the development of a tissue culture method of assay.

MATERIALS AND METHODS

Virus Isolates:

The standard Moredun isolate of virus (Li/31) was employed for laboratory manipulations. Two recent isolates of louping-ill virus (SB/526 and SB/527) which had been recovered by inoculating brain suspensions of clinically affected sheep i.c. into mice

(Brotherston and Boyce, personal communication) were used for challenge experiments.

Virus Stocks:

The Li/31 isolate was made available in glass sealed ampoules at the 5th i.c. pass level in three-week-old mice. Litters of one-day-old, random bred, Swiss white mice were inoculated with this material at a dilution of 10^{-3}. Inoculation was achieved by inserting a 26 gauge needle subcutaneously (s.c.) behind the head and depositing 0.01 ml into the cranium and 0.02 ml s.c. The brains of the surviving mice were harvested on the 5th day when the majority of mice were clinically affected. They were homogenised using a Teflon grinder[1.] as a 10^{-1} suspension in growth medium (see below). The homogenates were clarified by centrifugation at 1800 g and stored at -70° C in glass sealed ampoules. On each occasion that virus was required two ampoules were thawed, mixed and diluted in growth medium.

Virus inocula for challenge experiments were prepared similarly except that 10% inactivated horse serum saline (HSS) was used to homogenise the brains. In the first experiments stocks of SB/526 and SB/527 were made from mice that had been

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1. Camlab, Cambridge, England.

inoculated at three weeks of age with the original sheep brain. In subsequent experiments inoculum was prepared from brains of suckling mice that had been injected with SB/526. The mice were inoculated with a pool of blood collected from viraemic lambs that had been challenged with this isolate. This virus had, therefore, been isolated from sheep brain passaged once in three-week-old mouse brain, once in four-day-old lamb blood and once in one-day-old mouse brain. Normal mouse brains were homogenised and diluted for inoculation of control animals in the same way as the challenge inocula were.

Titration of virus in mice:

In the 1st series of experiments detection and titration of virus was performed in groups of five mice. Three-week-old mice were supplied from the Moredun stock of specific pathogen free, random bred, Swiss white mice (S/Swiss/ADR). Mice were inoculated into the left cerebral hemisphere using a 26 gauge needle and tuberculin syringe with 0.03 ml volumes. Mice were observed twice daily for 21 days for signs of louping-ill (Alston and Gibson, 1931 ; Greig et al, 1931) after which they were destroyed. All deaths occurring within the first three days were attributed to trauma. Clinically affected mice and mice found dead after the first three days were considered to be positive. Virus titres were calculated by the method of Reed and Muench (1938), and expressed as the $\log_{10} ID_{50}$ per 0.03 ml of the original material.

In all manipulations of materials for virus isolation care was taken to minimise loss of infectivity by thermal inactivation; all dilutions were made in diluents that had been chilled in an ice-bath.

Titration of virus in tissue culture

Medium:

Details of the media used for growth and maintenance are tabulated below:-

	Growth	Maintenance
Basic salt solution	Hank's	Earle's
Lactalbumin Hydrolysate[1.]	0.5% w/v	0.5% w/v
Yeast Extract[1.]	0.01% w/v	0.01% w/v
Folic acid	-	0.001 mg/ml
Inactivated bovine calf serum	10% v/v	5% v/v
Penicillin[2.]	100 i.u/ml)	200 i.u/ml)
Streptomycin[2.]	100 µ.g/ml)	200 µ.g/ml)
Mycostatin[3.]	-	100 i.u/ml

1. Difco Laboratories Ltd., Detroit, Michigan, U.S.A.

2. B.D.H., Poole, England.

3. E.R. Squibb & Sons, Liverpool, England.

The basic salt solutions (BSS) were prepared without glucose or sodium bicarbonate which were added immediately prior to use. Stock solutions of the other reagents were also prepared and added before use in the volumes indicated.

The overlay for plaquing consisted of maintenance medium containing 1.5% sodium carboxymethyl cellulose[1.] (CMC). This was achieved by adding 1.5 gm CMC to 64 ml of distilled water and standing overnight at + 4° C before autoclaving the suspension at 30 lbs for 20 minutes. The overlay was then prepared by adding 7.1 ml 10 x Earle's BSS together with the other constituents of the medium, immediately prior to use.

Cells:

The IB/RS2 clone 60 line of pig kidney cells obtained from the Animal Research Institute, Pirbright was employed and all experimental work was performed using cells between the 180th and 190th passage. This was accomplished by preparing ten Roux flasks from cells at the 176th passage level. On the fourth day when a confluent monolayer had developed the cells were detached with trypsin-versene (Madin and Darby, 1958) and suspended in growth medium containing 10% glycerine. The cell suspension was aliquoted in glass sealed ampoules and placed in an expanded polystyrene container at - 70° C in a mechanical freezer. Cultures were prepared periodically from the stored cells to maintain a constant supply of cells at the required passage level

1. Sodium carboxymethyl cellulose, Koch Light Laboratories Ltd., Colnebrook, England.

for experimental work.

Cells were grown in Roux flasks and passaged every five to six days at an expansion rate of 1:4. The medium was removed and the monolayer washed with phosphate buffered saline (PBS) pH 7.2 without the addition of calcium or magnesium (Dulbecco and Vogt, 1954). The cells were then treated with trypsin-versene at 37° C until the cells had detached. The cells from each flask were resuspended in fresh medium and distributed to a further four flasks.

Preparation of Monolayers:

Cells from four day old cultures were detached as described above and pooled in a conical flask containing a magnetic stirrer. A cell count was performed and the suspension adjusted to contain 3×10^5 cells/ml. Aliquots of 8 ml of this cell suspension were then distributed in 60 mm diameter plastic Petri dishes.[1] The plates were incubated over night at 37° C in a humidified atmosphere with 5% CO_2 by which time a confluent monolayer had formed.

Inoculation and Incubation:

The routine adopted was to decant the medium from the plates and then to add 0.2 ml of inoculum to each plate. These were

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1. Nunc, Algade S., Rosskilde, Denmark or Sterilin Ltd., Richmond, Surrey, England.

returned to the gassed incubator and rocked every 30 minutes for a two hour period of adsorption, prior to the addition of 6 ml of overlay. Unless otherwise stated plates were not washed following the period of adsorption. Plates were stained 80-90 hours post-inoculation with 1% (w/v) crystal violet[1.] in 20% (v/v) ethanol in distilled water.

RESULTS

Appearance of Plaques:

After 50-60 hours incubation small foci of rounded refractile cells were observed microscopically. The foci increased in diameter over the subsequent 24 hours and when stained appeared as distinct plaques 1.5 - 2 mm in diameter (Fig. 1:1).

The Effect of Dilution on the Plaque Count:

The relationship of the plaque count to the dilution of inoculum was investigated, by preparing dilutions of virus for inoculation onto groups of five plates each. There was a linear relationship between the number of plaques counted and the dilution of inoculum over a wide range and the linear regression correlation coefficient was + 0.9996 (Table 1:1; Fig. 1:2). The calculated

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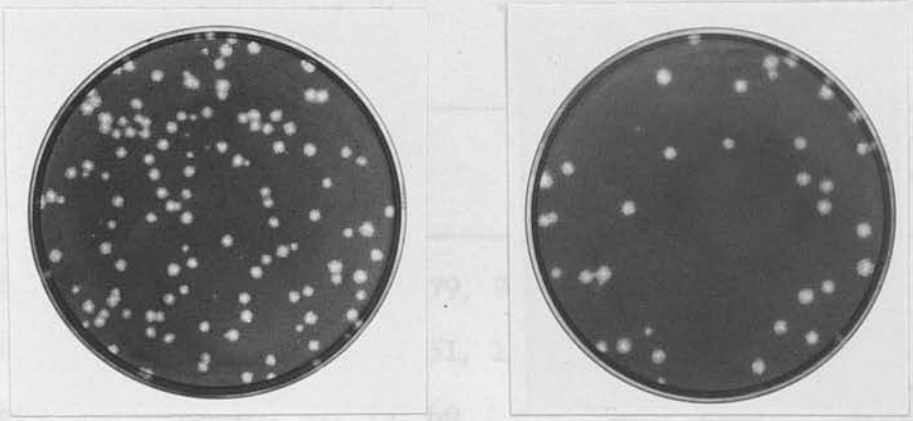
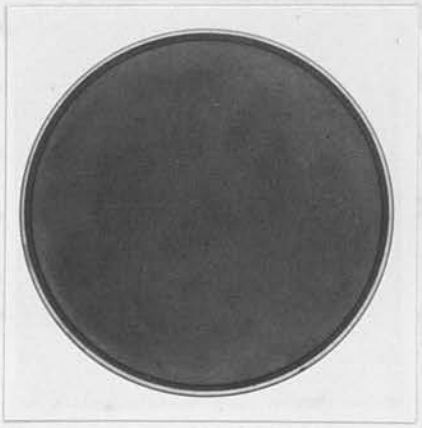
1. George T. Gurr Ltd., London, England.

Fig. 1:1. The appearance of louping-ill virus plaques on the pig kidney cell line IB/RS2 formed under sodium carboxymethyl cellulose and stained 80 hours post inoculation. A and B received dilutions of virus while C received control material (Actual size).



A B

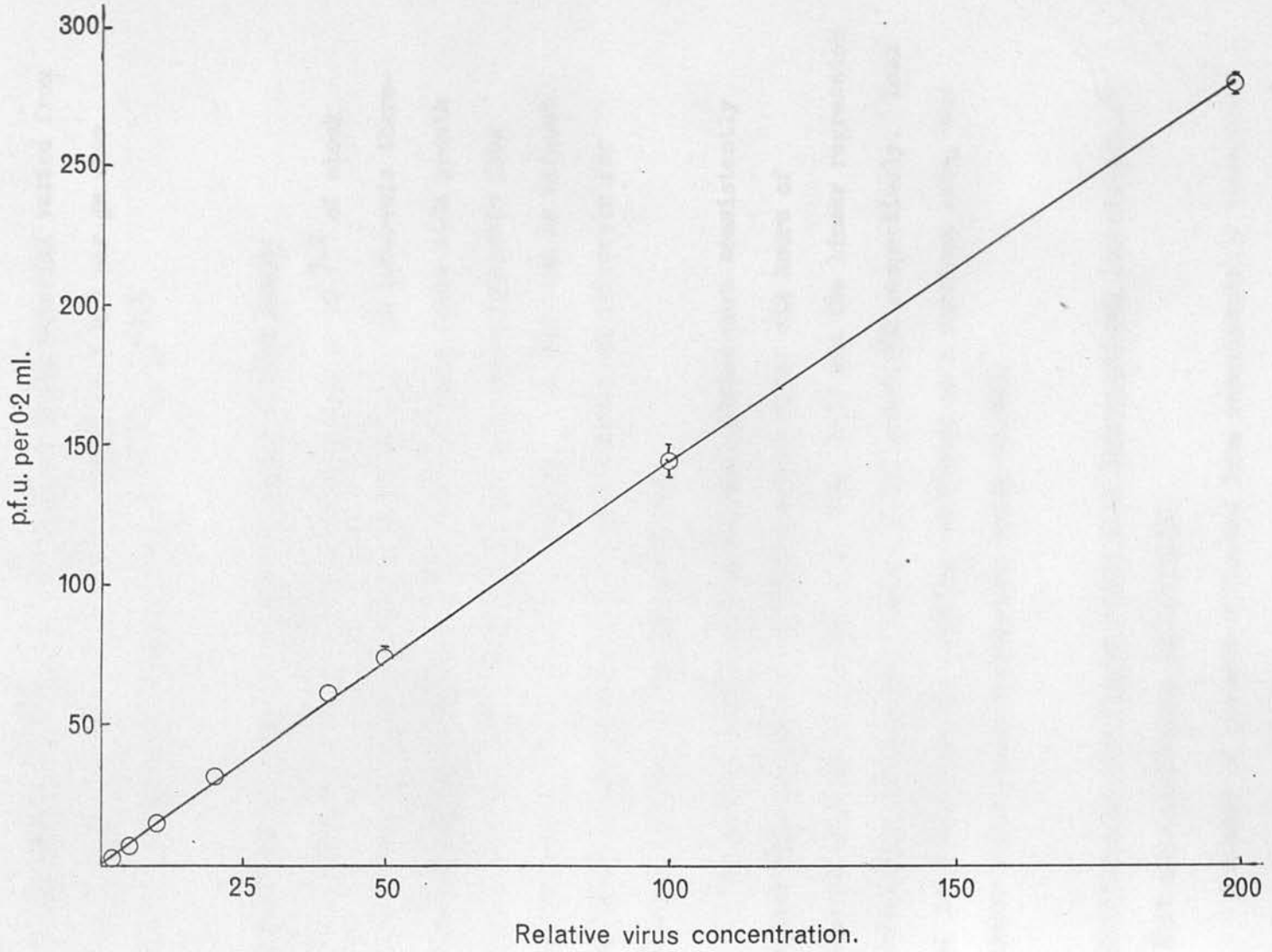


C

Table 1:1. The relationship of the number of plaque forming units to dilution using standard stock virus (Li/31).

Log_{10} dilution	Count	Mean $\pm$SE	Calculated titre log_{10}
6.3	265, 282, 278, 279, 283	277.4 $\pm$3.2	8.74
6.6	161, 134, 141, 151, 130	143.4 $\pm$5.7	8.75
6.9	70, 67, 91, 73, 69	74.0 $\pm$4.4	8.77
7.0	55, 66, 67, 56	61.0 $\pm$3.2	8.79
7.3	26, 38, 35, 26	31.3 $\pm$3.1	8.79
7.6	15, 12, 18, 16	15.3 $\pm$1.3	8.78
7.9	9, 8, 9, 5, 8	7.8 $\pm$0.7	8.79
8.0	9, 3, 4, 3, 7	5.2 $\pm$1.2	8.72
8.3	3, 4, 1, 3, 4	3.0 $\pm$0.6	8.78
8.6	0, 1, 0, 1, 3	1.0 $\pm$0.6	8.60

Fig. 1:2. The relationship of the number ($\pm$ SE) of plaque forming unit of louping-ill virus detected to the dilution of inoculum tested.


300
250
200
150
100
50
p.f.u. per 0·2 ml.
25
50
100
150
200
Relative virus concentration.

concentration of virus in the original brain material varied from $\log_{10}$ 8.60 to 8.79 plaque forming units (p.f.u.) per gm when tested at dilutions varying from $10^{-6.3}$ to $10^{-8.6}$.

The Effect of Time of Adsorption on the Plaque Count:

Plates were inoculated with a dilution of $10^{-7.2}$ of stock Li/31 virus and allowed to adsorb at 37° C. At intervals thereafter groups of five plates were washed three times with growth medium and then overlayed and at the same time intervals five plates were overlayed without washing. In addition an aliquot of inoculum was placed at 37° C and sampled at intervals for testing infectivity in the usual way.

The plaque counts in the unwashed plates were consistently higher than in the washed plates except after six hours of adsorption (Tables 1:2 and 1:3; Fig. 1:3) and the linear regression correlation coefficients were + 0.957 and+0.990 respectively. Loss of infectivity in the inoculum continued at a constant rate, the linear correlation coefficient being -0.993.

Comparison of the Plaque Assay with Intracerebral Inoculation of Mice for Determining Infectivity:

A number of plasmas collected from experimentally inoculated sheep were selected to cover a range of virus titres. Dilutions were prepared for mouse inoculation and for testing on tissue culture plates.

Table 1:2. The mean plaque count (±SE) from 5 plates following different periods of adsorption in plates that were washed prior to the addition of overlay and plates that were not washed.

Time of adsorption in hours	Washed	Not washed
1	25.6 ± 1.7	35.8 ± 2.7
2	34.8 ± 4.1	40.0 ± 1.7
3	39.5 ± 1.6	46.2 ± 2.7
4	42.6 ± 1.2	56.4 ± 2.4
5	56.2 ± 2.3	69.6 ± 2.9
6	64.0 ± 4.7	61.4 ± 3.6
7	66.0 ± 4.3	69.8 ± 6.2
8	77.0 ± 5.8	86.6 ± 3.7

Table 1:3. The mean plaque count ($\pm$ SE) from 5 plates of inoculum held at 37° C and tested after different intervals of time.

Time at 37° C in hours	Plaque count
0	40.0 $\pm$ 1.17
1	35.3 $\pm$ 1.88
2	33.2 $\pm$ 2.35
4	25.0 $\pm$ 3.08
6	20.5 $\pm$ 2.33

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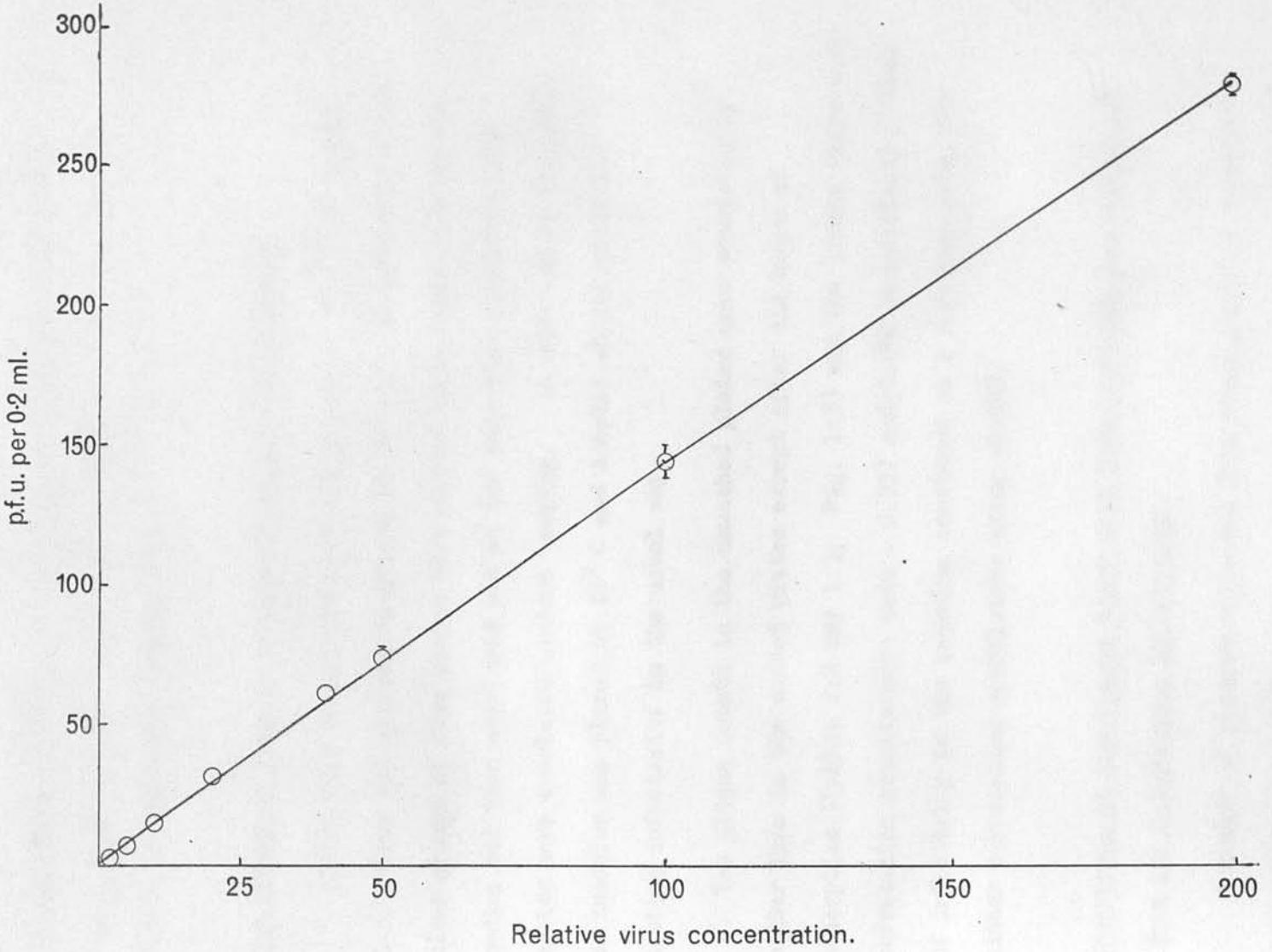
300
250
200
150
100
50
p.f.u. per 0·2 ml.
25
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100
150
200
Relative virus concentration.

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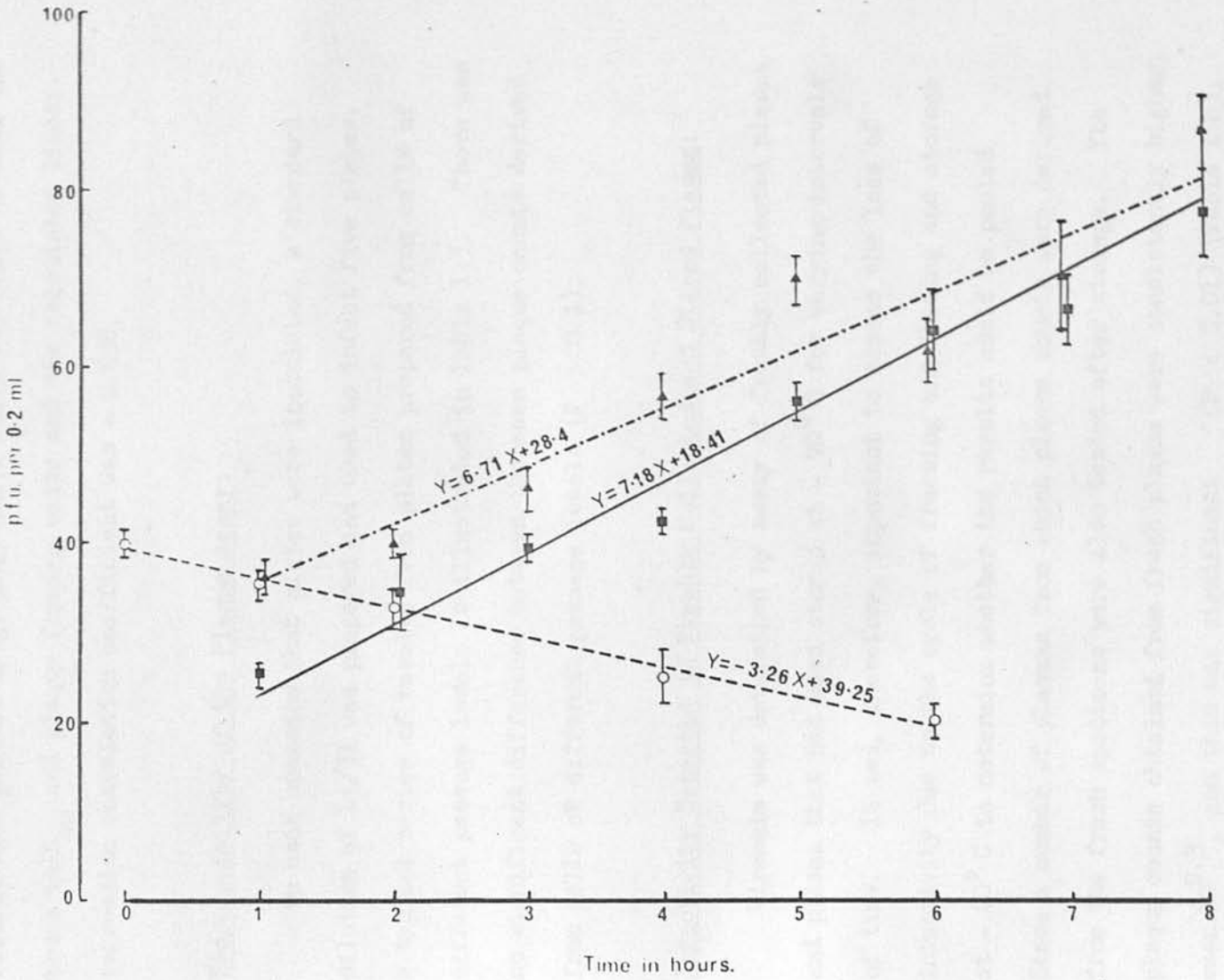
4	25.0 ± 3.08
6	20.5 ± 2.33

Fig. 1:3. The mean plaque count ($\pm$ SE) of louping-ill virus after different periods of adsorption with plates that were washed prior to the addition of overlay (——■——) were not washed (-·-·▲·-·-) and in the inoculum when held at 37° C prior to inoculation (- - -o- - -).


100
80
60
40
20
0
pfu per 0·2 ml
Y=6·71 X+28·4
Y=7·18X+18·41
Y=-3·26X+39·25
0
1
2
3
4
5
6
7
8
Time in hours.

When the results (Table 1:4) of the samples that gave positive results were plotted (Fig. 1:4) a 1:1 ratio was found between the mouse i.c. ID_{50} and plaque forming units and the calculated linear regression correlation coefficient was + 0.938.

Reproducibility of the Plaque Assay:

On each occasion that plates were inoculated, a standard dilution of Li/31 was prepared and used to infect five plates. A typical series of results from plates prepared from cells of different passage levels is illustrated in Table 1:5. There was no significant difference between the mean plaque counts derived from cells of different passage levels ($P > 0.1$).

Infectivity Detected in Freshly Collected and Stored Plasma:

Viraemia was determined by assay of freshly collected plasma and plasma that had been stored at - 70° C for varying intervals of time. It was, therefore, important to assess the loss of infectivity due to the cycle of freezing and thawing and storage at - 70° C to determine whether the results could be pooled. Hence a number of plasmas from which plaque counts were derived from the fresh specimens were also tested after storage. The plaque counts obtained from fresh plasma were consistently higher, mean $10^{0.3}$, and this was significant ($P < 0.01$) (Table 1:6).

Table 1:4. Results of testing sheep plasmas for virus collected from experimentally infected sheep by intracerebral inoculation of mice and by plaque assay.

Sheep No.	Day post-inoculation	Mouse i.c. ID_{50}		Plaque forming units	
		per 0.3ml	per 1ml	per 0.2 ml	per 1ml
3N13	1	Trace	Trace	(*86.0±11.0)x 10^0	2.63°
3N18	1	Trace	Trace	(110.0±11.1)x 10^0	2.74
3N34	1	1.5°	3.0°	(10.3± 1.7)x 10^1	2.71
3N41	1	N	N	N	N
3N45	1	1.3	2.8	(108.2±4.7)x $10^{0.4}$	3.13
3N34	2	2.8	4.3	(2.0±0.7)x 10^4	5.00
3N16	3	4.5	6.0	(4.5±1.8)x 10^4	5.35
3N33	3	3.2	4.7	(5.2±1.4)x 10^4	5.40
3N06	4	4.2	5.7	(5.8±0.7)x 10^4	5.46
3N31	4	5.2	6.7	(77.4±4.0)x 10^4	6.59
3N34	4	4.2	5.7	(121.0±4.2)x 10^4	6.78
3N38	4	5.0	6.5	(141.2±4.8)x 10^4	6.85
3N09	5	1.5	3.0	(23.8±3.3)x $10^{0.4}$	2.48
3N44	5	N	N	(3.6±0.8)x $10^{0.4}$	1.66
3N14	6	2.6	4.1	(151.0±4.4)x 10^1	3.88
3N16	6	N	N	N	N
3N20	6	N	N	N	N
3N38	7	N	N	N	N

° $\log_{10}$

* (Mean ± SE plaque count of 4 to 5 replicates) X dilution tested.

Fig. 1:4. The relationship of louping-ill virus infectivity as determined by intracerebral inoculation of mice and plaque assay, in sheep plasmas. Figures are expressed as $\log_{10}$ per ml.

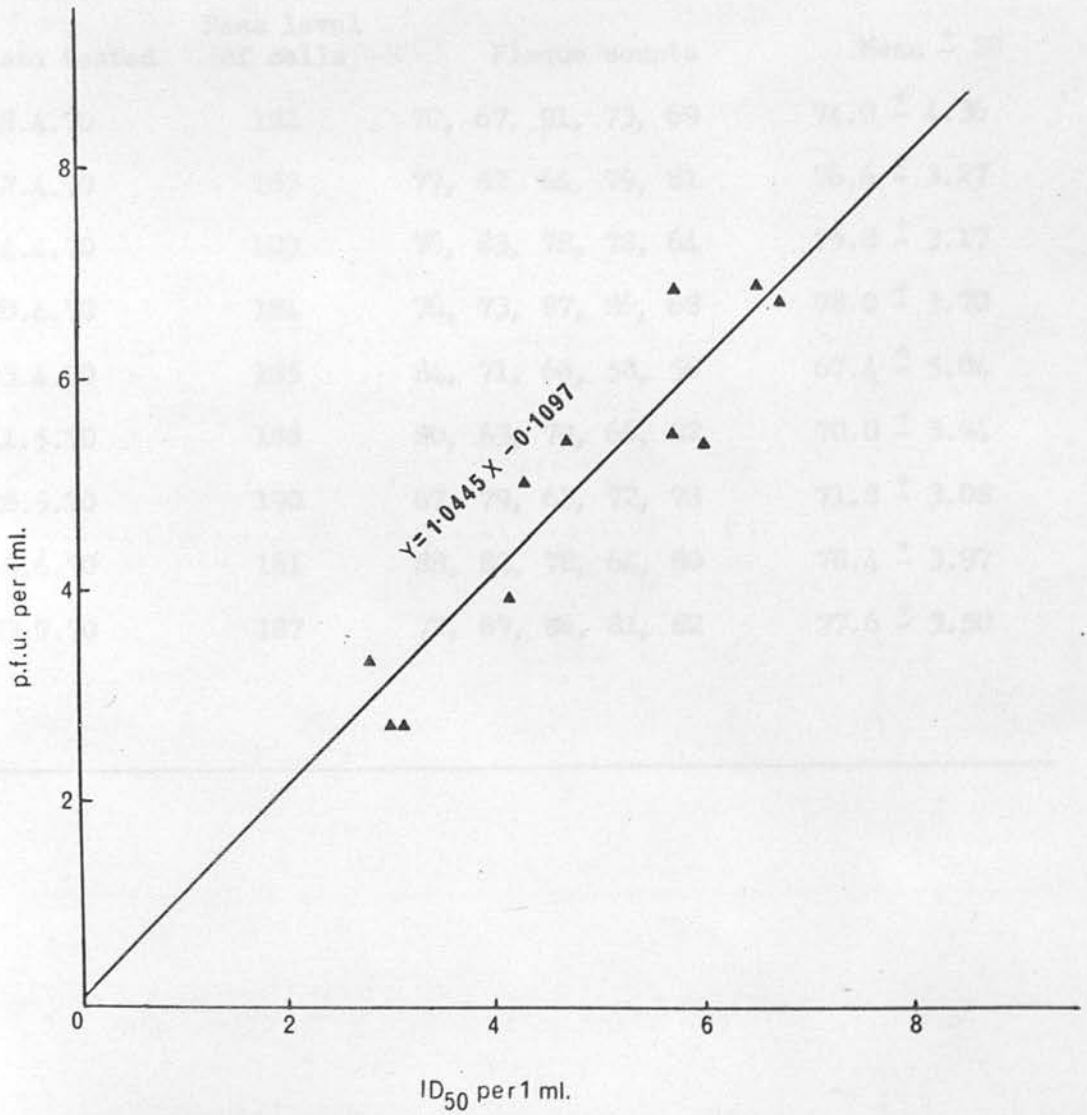

Y=1·0445 X −0·1097
p.f.u. per 1ml.
ID50 per 1 ml.
0
2
4
6
8

Table 1:5. Infectivity detected by plaque assay using a standard inoculum and cells at different pass levels.

Date tested	Pass level of cells	Plaque counts	Mean ± SE
8.4.70	182	70, 67, 91, 73, 69	74.0 ± 4.36
12.4.70	183	77, 82, 64, 79, 81	76.6 ± 3.27
14.4.70	183	76, 83, 78, 78, 64	75.8 ± 3.17
20.4.70	184	76, 73, 87, 86, 68	78.0 ± 3.70
23.4.70	185	84, 71, 68, 58, 56	67.4 ± 5.04
11.5.70	188	80, 63, 79, 66, 62	70.0 ± 3.94
28.5.70	190	67, 79, 63, 72, 78	71.8 ± 3.08
8.6.70	181	88, 82, 78, 64, 80	78,4 ± 3.97
23.7.70	187	72, 67, 86, 81, 82	77.6 ± 3.50

Table 1:6. Infectivity detected in sheep plasma freshly collected, and following storage at -70° C. Fresh plasma was tested undiluted and stored plasma at a dilution of $10^{-0.4}$.

Sheep No.	Day post-inoculation	Fresh Count	Fresh Titre	Period of Storage (months)	Stored Count	Stored Titre
3N02	4	⊕26.8±3.4	*1.43±0.53	6	⊕5.2±1.4	*1.12±0.55
3N06	1	20.8±3.6	1.32±0.56	6	2.6±0.5	0.82±0.04
3N07	1	106.0±8.7	2.02±0.94	6	22.4±2.0	1.75±0.70
3N20	1	79.0±4.6	1.90±0.66	3.5	20.6±1.8	1.71±0.26
3N21	1	139.3±11.9	2.14±1.08	6	31.8±3.3	1.90±0.92
3N29	1	86.5±8.2	1.94±0.91	3.5	35.8±1.7	1.95±0.63
3N31	1	58.7±2.2	1.77±0.34	6	8.6±1.0	1.33±0.40
3N47	4	39.8±4.6	1.60±0.66	6	7.6±1.1	1.28±0.44

⊕ The mean number of plaque forming units ±SE

* Titre expressed as $\log_{10}$ plaque forming units per 0.2 ml of plasma.

DISCUSSION AND CONCLUSIONS

Since the original development of plaque techniques for assay of animal viruses in tissue culture (Dulbecco, 1952) this method has been used extensively for precise quantitation of infectivity of numerous viruses including 205 arthropod transmitted togavirus serotypes (Stim, 1969). The louping-ill virus plaques that developed using the system outlined above were clear and readily counted. In addition there was a linear relationship between the number of plaques observed and the concentration of virus tested which confirmed that each plaque originated from a single virus particle defined as the virus unit unseparable by dilution (Dulbecco and Vogt, 1954).

When the plaque counts were compared in plates that were washed and plates that were not washed it was apparent that washing removed unadsorbed virus, some of which may adsorb subsequent to the addition of overlay. In both sets of plates there was a relatively constant increase in the plaque count with time of adsorption. Residual unadsorbed virus in the inoculum would have been depleted due to both adsorption and thermal inactivation. A proportion of the increase in the plaque counts detected in the inoculum in the plates adsorbed for the greater periods of time may, therefore, have been due in part to leakage of virus following a first cycle of replication. From these results it was not, therefore, possible to determine the period of adsorption that

would give maximum efficiency of plaquing prior to the release of virus. A period of two hours adsorption was, however, adhered to in all experimental work.

Comparison of the mouse i.c. ID_{50} and the plaque test for detecting infectivity indicated that there existed a 1:1 relationship. The plaque test was, therefore, at least as sensitive as the i.c. inoculation of mice for the detection of virus. In addition the plaque method was found to be highly reproducible, there being no significant difference in the plaque counts determined in a standard inoculum when tested using cells at different passage levels over a period of time. The plaque method, therefore, compared favourably with the use of mice for virus assay as regards to sensitivity. Moreover, the method was found to be considerably cheaper and less time consuming than the mouse test; when it became available it was adopted for all assay purposes.

On average the infectivity detected in fresh plasma was two fold higher than in plasma that had been stored. This loss of infectivity was presumably due to the cycle of freezing and thawing. Although this loss was consistently present it was slight and no account was taken of it in analysis of experimental results.

CHAPTER 2

MATERIALS AND METHODS EMPLOYED: 2 ANTIBODY ASSAY

INTRODUCTION

The haemagglutination-inhibition (HI) test as described by Clarke and Casals (1958) had been in routine use at Moredun for a number of years when this study commenced, and it was the practice to extract non-specific inhibitors with acetone which is both expensive and time consuming. The method employing acid washed kaolin was therefore considered as an alternative.

In addition to providing a method of great value for accurately enumerating infectivity, plaque methods may also be used for accurate and sensitive antibody assay (Dulbecco, Vogt and Strickland, 1956; Russell, Nisalak, Sukhavachana and Vivona, 1967). The possibility of assaying the development of neutralizing antibody in sheep employing the plaque method was therefore investigated.

Webb and Smith (1966) have suggested that the class of antibody present during a togavirus infection might be an important factor in the pathogenesis of infection. It was, therefore, considered pertinent to apply methods that would permit this feature of infection to be investigated. Jonas (1969) described two simple methods for determining the class of antibody in sheep serum which were adapted for the present work.

Webb, Connolly, Kane, O'Reilly and Simpson (1968a) have produced evidence that during louping-ill infection of man antibody to the virus was locally produced in the central nervous system (CNS). Their evidence rested on the fact that the ratio of antibody in serum and cerebrospinal fluid (CSF) was considerably higher to an antigen not involved in the disease process than it was to louping-ill virus. In order that this feature of infection might be investigated a serum antibody other than that to louping-ill virus was required. The possibility of using antibody to egg albumen for this purpose was therefore investigated.

Evidence of local synthesis of γ-globulin in the CNS had also been found in studies of a number of conditions of man other than louping-ill virus encephalitis many of which have relied on quantitation of globulin fractions present in the CSF (for review see Heremans, 1968). Assay systems for ovine IgG and IgM were therefore developed.

The development and adaptation of these methods are described in the present chapter.

MATERIALS AND METHODS

The Haemagglutination-Inhibition Test:

The reagents for the test were prepared as described by Clarke and Casals (1958). Antigen was derived by the sucrose-acetone-ether method from sucking mice brains infected with Li/31.

Following deproteinization with protamine sulphate such antigens contained between 512 and 2,048 haemagglutinating units per 0.4 ml and were adjusted for the test to contain 4-8 haemagglutinating units per 0.2 ml. Haemagglutinating activity was detected using standard, gander, red-blood cell suspensions of 0.24% (v/v) prepared by estimating the optical density using a spectrophotometer. Sera were adsorbed prior to testing with a drop of sedimented red cells.

Routinely dilutions of serum and antigen were made in two-fold steps in standard WHO leucite haemagglutination plates. The antigen antibody mixtures at pH 9.0 reacted overnight at + 4° C and the red cells were added in diluent to adjust the pH to 6.2. End points were assessed on a 50% basis after an incubation period of one hour at room temperature.

Comparative tests using both the kaolin[1.] and acetone methods for extracting non-specific haemagglutination-inhibitors (NSHI) indicated that both methods were equally effective. Kaolin was therefore used for extracting sera routinely.

Extraction of CSF was investigated using paired serum and CSF from four animals that had been inoculated with louping-ill virus and egg albumen, and four animals that had been inoculated with egg

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1. Hopkin and Williams Ltd., Chadwell, Essex.

albumen alone. HI activity to louping-ill virus and passive haemagglutination (PHA) activity to egg albumen was determined in the samples before extraction and following extraction with kaolin and acetone. The initial $^{1}/_{10}$ dilution of CSF prior to extraction with acetone was made with 0.85% saline containing 0.5% bovine albumin (BA) and the $^{1}/_{5}$ dilution prior to kaolin extraction was made with 1% BA in borate buffered saline; thus by either method the final concentration of BA was approximately 0.5%.

Unextracted CSF was tested at an initial dilution of $^{1}/_{2.5}$ while all other specimens were tested at an initial dilution of $^{1}/_{10}$.

Inactivation of IgM Class Immunoglobulin:

Test acute sera were obtained from either lambs naturally affected with louping-ill virus or sheep and lambs that had been infected experimentally. Hyperimmune serum was collected from a sheep (7H 92) that had been vaccinated (Brotherston and Boyce, 1969) and subsequently challenged with SB/526 by the i.c. route. Negative sera were collected from sheep that had been inoculated with control mouse brain homogenate.

The HI antibody titres of sera were determined following treatment with 2-mercaptoethanol (2-ME) and heat and compared with those of the untreated specimens. The optimal condition for 2-ME treatment was investigated using a range of molarities prepared in PBS (pH 7.2). One volume of 2-ME solution were added to nine

of either unextracted sera or sera that had been extracted. As the process of extraction of sera involved a 1/10 dilution the molarity of 2-ME solution added to such specimens was 1/10th that added to whole serum. Treatment with 2-ME was allowed to continue overnight at + 4° C and as 2-ME was found not to affect the HI test, specimens were not dialysed before testing for HI activity. Sera were also subjected to a range of temperatures for 30 minutes both before and after extraction.

Immunoelectrophoresis:

Confirmation that the treatments employed were destroying IgM was sought using immunoelectrophoresis (IEP). This was performed as described by Schiedegger (1955) using Oxoid purified agar[1.] in Veronal-HCl buffer (pH 8.2) ionic strength 0.05 on 3" x 1" microscope slides. Origins are charged with 5 µl and electrophoresis was maintained for 100 minutes with a potential difference of 150 V and a current of 3 to 4 m.a. per slide. The troughs were then charged with 0.1 ml of antisera and allowed to react for 48 hours. After the slides had been soaked in 0.85% saline for 48 hours they were dried and stained in 0.1% azocarmine-B and fixed with 5% acetic acid. Precipitin lines were identified using the

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1. Oxoid Ltd., London, England.

nomenclature recommended by the WHO Committee on Immunoglobulins (1964).

Sephadex G-200 filtration:

Fractionation of sera using Sephadex G-200 was employed to confirm the results of treating sera with 2-ME and heat. Aliquots of the acute sera were treated with either heat or 2-ME prior to extraction with acetone. Extraction was performed as described above, except that the final precipitates were resuspended to the original volumes of serum in tris-HCL buffer (0.1 M tris (hydroxymethyl) 1.0 M sodium chloride) (pH 8.0). The extracted samples were passed separately through a Sephadex G-200 column and the HI activity in the eluting fractions was examined.

Equipment used for Fractionation:

Column fractionation was performed using the following equipment. A Watson Marlow flow inducer[1.] was used at a rate of 25 ml/hour for Sephadex G-200 filtration and at 40 ml/hour with ion exchange columns. Protein present in eluates was continuously monitored using a LKB Uvicord II,[2.] reading absorbancy at 280 n.m., connected

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1. Watson-Marlow Air Pump Co., Bucks, England.

2. LKB Instruments Ltd., Bromma, Sweden.

to a Vitatron linear recorder (Type UR 404).[1.] Four ml. samples of eluate were collected in tubes placed in a LKB Ultrarack (Type 7000).[2.] Concentration of fractions were achieved by force dialysis using Sartorius Collodium Bags (Type 13200, pore size 10 μm)[3.], with a pressure of 500 mm of mercury.

Separation of serum fractions:

The methods adopted for the separation of immunoglobulins were based on the techniques of Aalund, Osebold and Murphy (1964). IgG was obtained by fractionation of pooled sheep serum on Whatman DE-32 ion exchange cellulose[4.] which had been equilibrated with 0.02M PO_4 (pH 7.4). Thirty ml of serum was dialysed overnight against the same buffer and applied to the top of a column[5.] (1.5 x 25 cm). The fall through peak was concentrated against tris-HCl buffer to 7 ml and then applied in 3.5 ml aliquots to a Sephadex G-200 column (2.5 x 73 cm) which had been equilibrated with

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1. Vitatronon, Dieren, Holland.

2. LKB Instruments Ltd., Bromma, Sweden.

3. Sartorious Membranfilter GmbH., Grottingen, Germany.

4. Balston Ltd., Maidstone, England.

5. Pharmacia Fine Chemicals Ltd., Uppsala, Sweden.

the same tris buffer. One major peak and two secondary peaks eluted (Fig. 2:1). The centre of the major peak from each filtration was concentrated and pooled to give a final volume of 4 ml. This material was recycled through the G-200 column and a single symmetrical peak eluted, which was concentrated against PBS (pH 7.4) to a final volume of 8 ml before being lyophilized and stored at -70° C.

For the separation of IgM, euglobulins were prepared by diluting 2 litres of pooled sheep serum 1:20 in distilled water. The diluted serum was left overnight and centrifuged at 1800 g for 60 minutes at 4° C. The resulting precipitate was resuspended in tris-HCl buffer to give a final volume of 11 ml, which was clarified at 1800 g for 20 minutes. One ml samples were applied to a Sephadex G-200 column (2.5 x 57 cm) which had been equilibrated with the same tris buffer. Two well separated peaks emerged (Fig. 2:2). The first half only of the earliest exclusion peaks from these filtrations were pooled and concentrated to 10 ml against 0.075 M PO_4 (pH 8). The concentrate was applied to a DEAE Sephadex A50 (particle size 40-120 μ) column (1.5 x 25 cm) which had been equilibrated with the same buffer. A small peak emerged when the column was washed with 50 ml of 0.125 M PO_4. Finally, 50 ml of 0.2 M PO_4 was run on and a single large peak eluted (Fig. 2:3). The whole of this peak was concentrated against PBS (pH 7.4) to 4 ml and stored at - 70° C in sealed ampoules.

Antisera were raised to the serum fractions and to whole sheep

Fig. 2:1. Protein distribution of sheep serum following Sephadex G-200 filtration of the fall through peak from a Whatman DE/32 ion exchange column equilibriated with 0.02M PO_4 (pH 7.4).

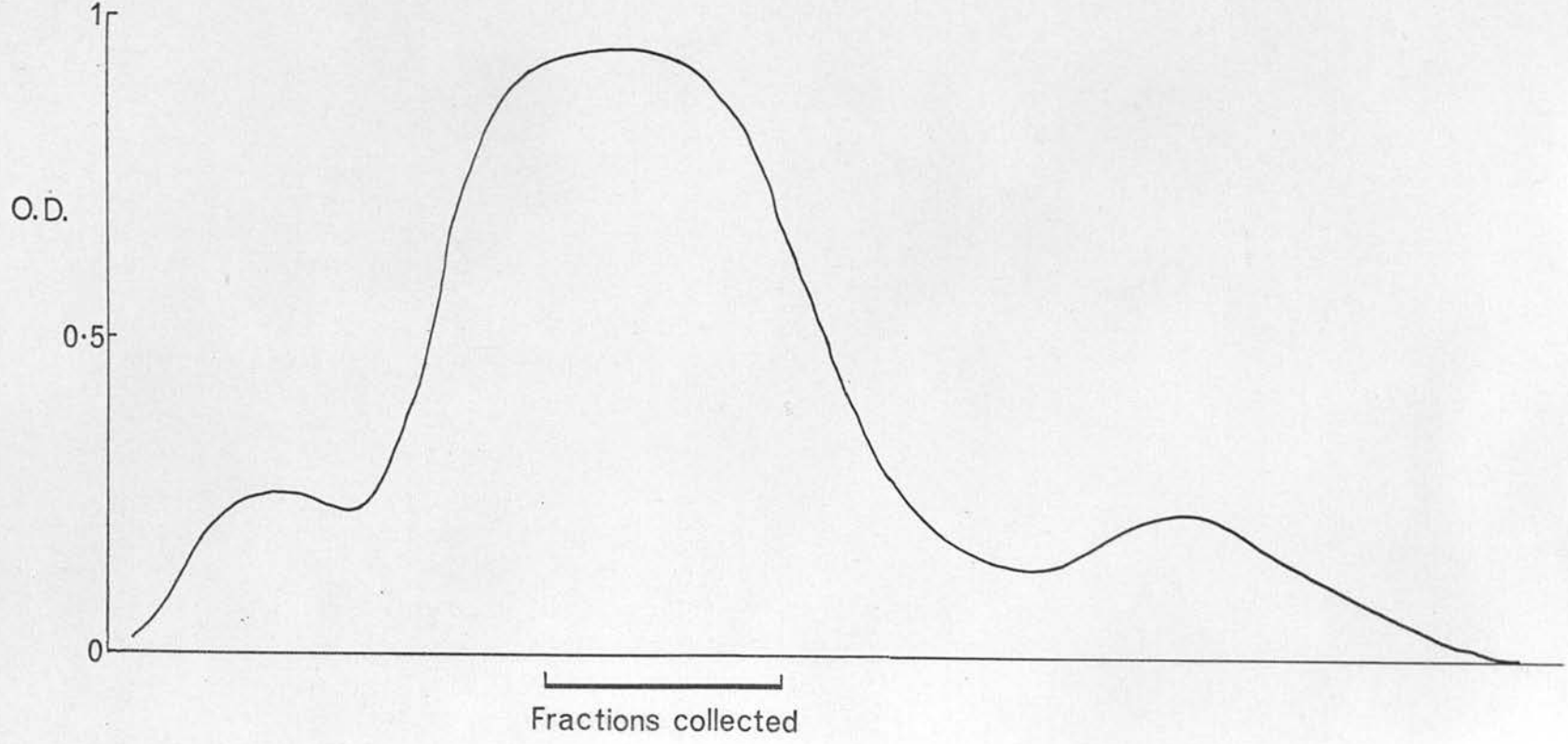

1
O.D.
0·5
0
Fractions collected

Fig. 2:2. Protein distribution of sheep euglobulin following Sephadex G-200 filtration.

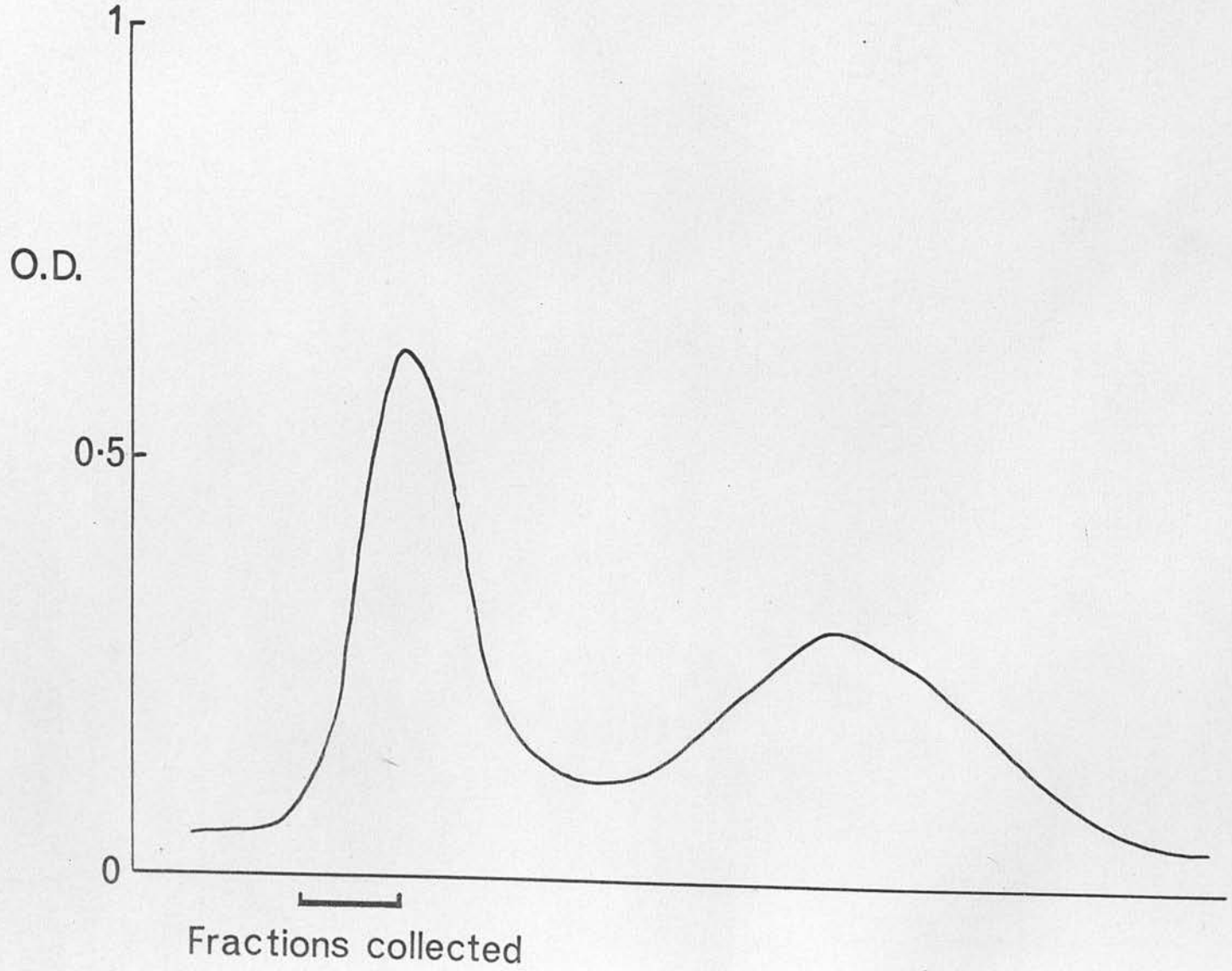
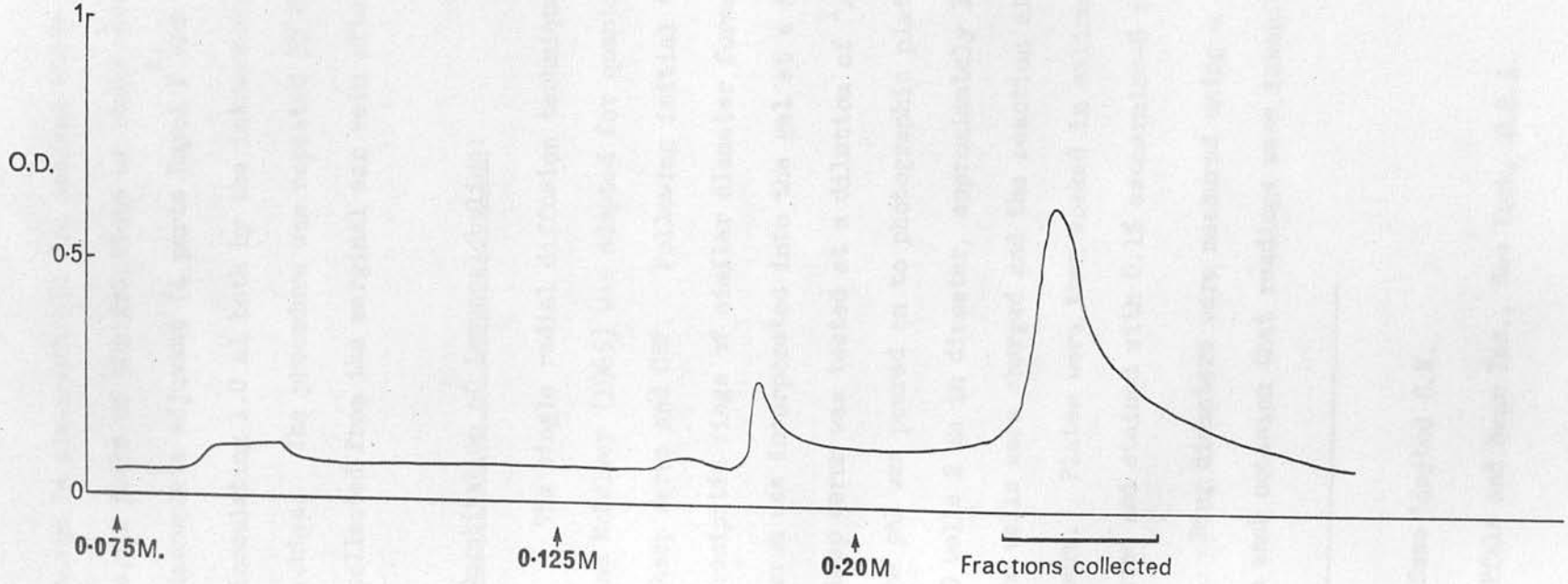

1
O.D.
0·5
0
Fractions collected

Fig. 2:3. Elution of sheep serum protein from a DEAE Sephadex A-50 column following application of the first half of the earliest exclusion peak from Sephadex G-200 filtration of the euglobulin fraction.


1
O.D.
0·5
0
0·075M.
0·125M
0·20M
Fractions collected

serum in six-month-old New Zealand White rabbits by emulsifying equal parts of the fractions or whole sheep serum with Freund's incomplete adjuvant (4 parts Bayol F[1.] and 1 part Falba[2.]) and inoculating 1.0 ml both by the intravenous and by the subcutaneous routes. The procedure was repeated 30 days later and serum was collected from the marginal ear vein after a further 10 days.

Quantitation of immunoglobulins:

The single radial diffusion technique as modified by Fahey and McKelvey (1965) was adapted for quantitating IgG and IgM in sheep serum and CSF. Following initial tests it was found that precipitin rings of similar diameter formed when the rabbit anti-IgG serum was incorporated into the gel at a final dilution of $^{1}/_{16}$ and sheep serum was tested at a dilution of $^{1}/_{200}$ and CSF undiluted. The gel was poured on to photographic plates (10.7 x 8.2 cm) and 20 wells 2 mm in diameter, approximately 1.7 cm apart were cut. The wells were charged and the reaction allowed to continue for 24 hours. Plates were then soaked in saline for 48 hours and then dried and stained with 0.1% azocarmine-B in 5% acetic acid.

Ring diameters were measured using a photographic enlarger. On each occasion that readings were taken the magnification was

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1. Esso Limited U.K.

2. Pfalz and Baur Inc., New York, U.S.A.

adjusted to the same level using a calibrated glass slide and the images of the precipitin rings were measured using steel dividers and ruler.

Initial tests indicated that no IgM was detectable in control CSF or in such CSF concentrated 43 times by force dialysis. It was, therefore, necessary to determine the conditions of the test, using CSF collected from animals that were acutely affected with louping-ill. Comparative precipitin rings were obtained when the rabbit anti-IgM was incorporated into the gel at a final concentration of 1/64 and wells were cut 3 mm in diameter. Sheep sera were tested at a dilution of 1/20 and CSF undiluted. The reaction was allowed to proceed for 48 hours before washing and staining the plates as described above.

Antibody to egg albumen:

For detecting antibody to egg albumen the PHA test was performed as described by Herbert (1967). Stock egg albumen[1] was prepared as a clarified 20% w/v solution which was used for inoculation of sheep and to give a solution of 0.25% w/v for sensitizing cells. Fresh tannic acid was prepared on each occasion as a 0.01% w/v solution. Sheep cells were collected on the day of the test or 24 hours before testing, and all the tests were conducted using cells from one sheep. Sheep red cell adsorbed horse serum was used to stabilize the reaction at a 1% concentration in PBS (pH 7.2).

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1. B.D.H., Poole, Dorset, England.

The distribution of antibody to egg albumen in serum and CSF was investigated using sera and CSF collected from nine sheep which had been vaccinated some months previously with a commercially available vaccine prepared from infected egg-embryos. In addition the antibody response to egg albumen was investigated in seven sheep. The sheep were inoculated with either 10 ml, 5 ml, 2 ml, or 1 ml of the 20% egg albumen solution and were bled on day 5, 10 and 20 for serum.

Neutralizing antibody:

Serum neutralizing antibody was investigated using sera from animals that had received either Li/526 or control material. All sera were inactivated at 56° C for 30 minutes prior to testing. Initial tests indicated that optimal plaque reduction occurred when serum virus mixtures were incubated together for a period of 30 minutes at 37° C; these conditions were adopted for all tests.

The influence of complement (C′) on specific and non-specific neutralization was investigated using fresh guinea pig serum at a dilution of 1/10. Serum and virus dilutions were prepared in growth medium and the concentration of virus was adjusted to give approximately 70 plaques in control mixtures. Re-action mixtures consisted of 1 volume each of serum, medium and virus. The aliquot of medium was replaced by a similar volume of 1/10 guinea pig serum in tests for the action of C′. Control mixtures consisted of two volumes of medium and one of virus. Following reaction serum virus

mixtures were each inoculated on to groups of five culture plates which were allowed to adsorb for two hours. The plates were then washed twice with medium prior to the addition of overlay.

Interference:

Interference due to inactivated louping-ill virus was investigated using stock virus at a dilution of 10^{-2} which had been heated at 56°C for 30 minutes to inactivate virus, allowed to cool and then mixed with an equal volume of stock virus at a dilution of $10^{-7.7}$. Control mixtures containing either an equal volume of medium or similarly treated normal mouse brain were prepared likewise. Infectivity in the preparations was then assayed.

RESULTS

Extraction of non-specific inhibitors:

The titres of NSHI in four negative sera were between $^{1}/_{1280}$ and $^{1}/_{2560}$, which effect was completely removed following extraction with acetone or kaolin. The pattern of the pellicle formed at the lowest dilution of the extracted specimens ($^{1}/_{10}$) showed no trace of H I. There was no consistant difference in the titres of the same positive serum following extraction by either method (Table 2:1). The titres detected in unextracted and extracted CSF samples were identical but the titres in the kaolin extracted samples were consistently lower. The highest level of NSHI detected

Table 2:1. Reciprocal titres of unextracted, acetone-extracted, and kaolin-extracted sera and CSF using the Haemagglutination-Inhibition Test (HI) and the Passive Haemagglutination Test (PHA).

	HI						PHA					
	SERA			CSF			SERA			CSF		
Specimen No.	U	A	K	U	A	K	U	A	K	U	A	K
1	10240	1280	> 640	80	80	20	640	320	640	5	40	40
2	10240	>2560	2560	40	40	10	640	320	320	2.5	40	40
3	>5120	>640	1280	160	160	80	80	80	80	2.5	40	40
4	2560	320	320	160	160	40	80	80	80	<2.5	40	40
5	1280	<10	<10	2.5	<10	<10	80	80	80	<2.5	40	40
6	2560	<10	<10	<2.5	<10	<10	80	80	80	<2.5	40	40
7	1280	<10	<10	<2.5	<10	<10	10240	10240	20480	80	80	40
8	2560	<10	<10	<2.5	<10	<10	2560	2560	1280	2.5	80	40

Specimens 1 - 4 were collected from animals inoculated with egg albumen + louping-ill virus.

Specimens 5 - 8 were collected from animals inoculated with egg albumen alone.

U = Unextracted

A = Acetone extracted

K = Kaolin extracted

in CSF was $^{1}/2.5$.

Antibody titres to egg albumen detected in whole serum and serum extracted by either methods were of the same order. No antibody was detected in CSF in four samples tested before extraction, while three had low levels and one had a titre of 80. However, samples extracted by either method caused consistent agglutination up to a titre of $^{1}/40$ to $^{1}/80$.

Inactivation of IgM:

Neither heat treatment at 62.5° C nor treatment with 1 M 2-ME affected the titres of hyperimmune serum while activity in pools of acute phase sera was (Table 2:2). The reduction recorded in the acute sera was, however, greater when treated prior to extraction than when extracted samples were treated. Optimal conditions of heat and 2-ME treatments were, therefore, investigated using unextracted sera.

Temperatures less than 60° C had no effect on the titre of a diagnostic serum and treatment at 62.5° C caused a one-fold reduction in titre while treatment at 64.5° C caused a four-fold decrease (Table 2:3). The activity in the hyperimmune serum was reduced by less than 1 well when it was treated at this temperature. Sera treated with 4 M 2-ME tended to gel; sera treated with 2 M or 1 M solutions did not. The titre of the diagnostic serum was reduced with all molarities of 2-ME employed, however, the reduction brought about the 2 M solution was greater than caused by a 1 M solution;

Table 2:2. The reciprocal haemagglutination-inhibiting antibody titres in sheep serum following treatment with 1 M 2-Mercaptoethanol and heat (62.5° C).

Serum	Extracted before treatment			Extracted after treatment		
	Nil	2-ME	Heat	Nil	2-ME	Heat
Hyperimmune (7H92)	>1280	1280	>1280	>1280	>1280	1280
10 days post-inoculation	1280	>40	160	>640	80	>10
15 days post-inoculation	>320	320	320	640	160	>40
Control	<10	<10	<10	<10	<10	<10

Table 2:3. Reciprocal haemagglutination-inhibiting antibody titre in sheep serum following treatment at varying temperatures and different molarities of 2-Mercaptoethanol.

Treatment		Diagnostic	Hyperimmune	Control
4° C		>2560	1280	<10
56° C		>2560	1280	<10
60° C		>2560	1280	<10
62.5° C		>1280	>640	<10
64.5° C		160	>640	<10
2-ME	PBS	>2560	>1280	<10
	1M	320	>1280	<10
	2M	160	<2560	<10
	4M	10+	1280	<10+

+ Serum gelled.

the hyperimmune serum was not affected by 2-ME at these molarities.

Typical IEP reactions of sheep sera are illustrated in Fig. 2:4. The IgM precipitin lines, arrowed, were clearly defined when reacted with all antisera except that raised against IgG, but IgM was not detected in serum treated with either 1 M or 2 M 2-ME (Fig. 2:5). When sera treated with 2-ME were reacted with anti-IgM a distinct line appeared which was slightly altered in position and diffused more rapidly than IgM, and probably represented the disrupted fragments of IgM. Following treatment at 62.5° C a faint IgM precipitin line was still discernible (Fig. 2:6). However, when treated at 64.5° C the IgM line was absent as was the α-macroglobulin fraction. The IgG precipitin line remained unaltered after all treatments.

The distribution of HI activity in the eluates of an acute phase serum following Sephadex G-200 filtration is shown in Fig. 2:7. The activity in the untreated sample was distributed between the first and second peak; following heat or 2-ME treatment activity was entirely restricted to the second peaks. No activity was detected in the eluates of similarly treated negative sera.

Quantitation of immuno-globulins:

The final preparation of IgG contained 23.44 mg of protein per ml by Kjeldahl analysis and on IEP a single precipitin line developed, which corresponded in position and appearance to IgG when reacted against rabbit anti-whole sheep serum.

Fig. 2:4, 2:5, 2:6. Immunoelectrophoresis of whole sheep serum and serum subjected to heat and 2-Mercaptoethanol treatment reacted with:
A: rabbit anti-sheep euglobulin, B: rabbit anti-whole sheep serum, C: rabbit anti-IgM adsorbed with IgG, D: rabbit anti-IgG.
The arrows indicate the IgM precipitin line.

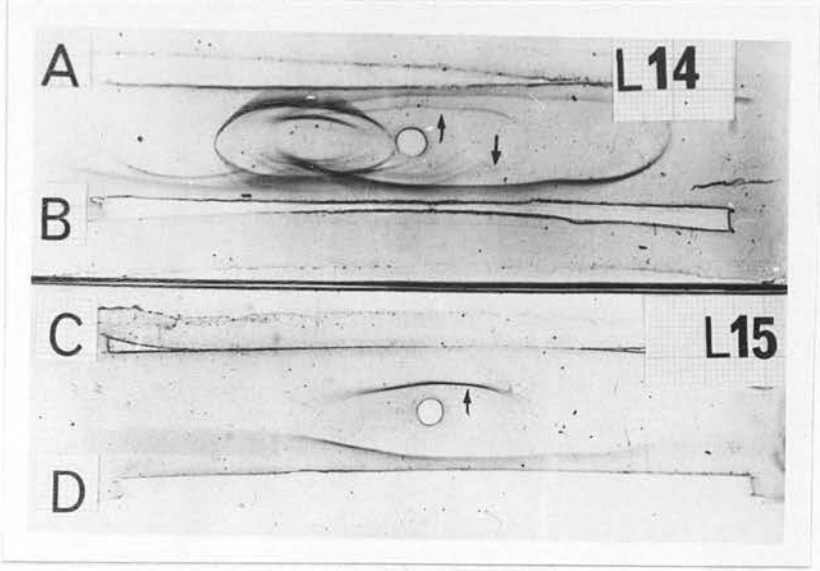


Fig. 2:4. Origins charged with whole sheep serum.

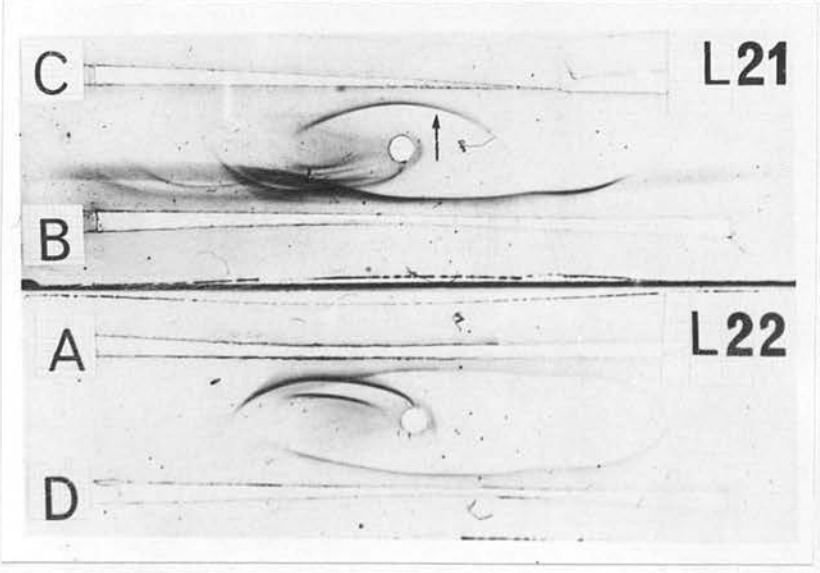


Fig. 2:5. Origins charged with sheep serum following treatment with 2M 2-Mercaptoethanol.

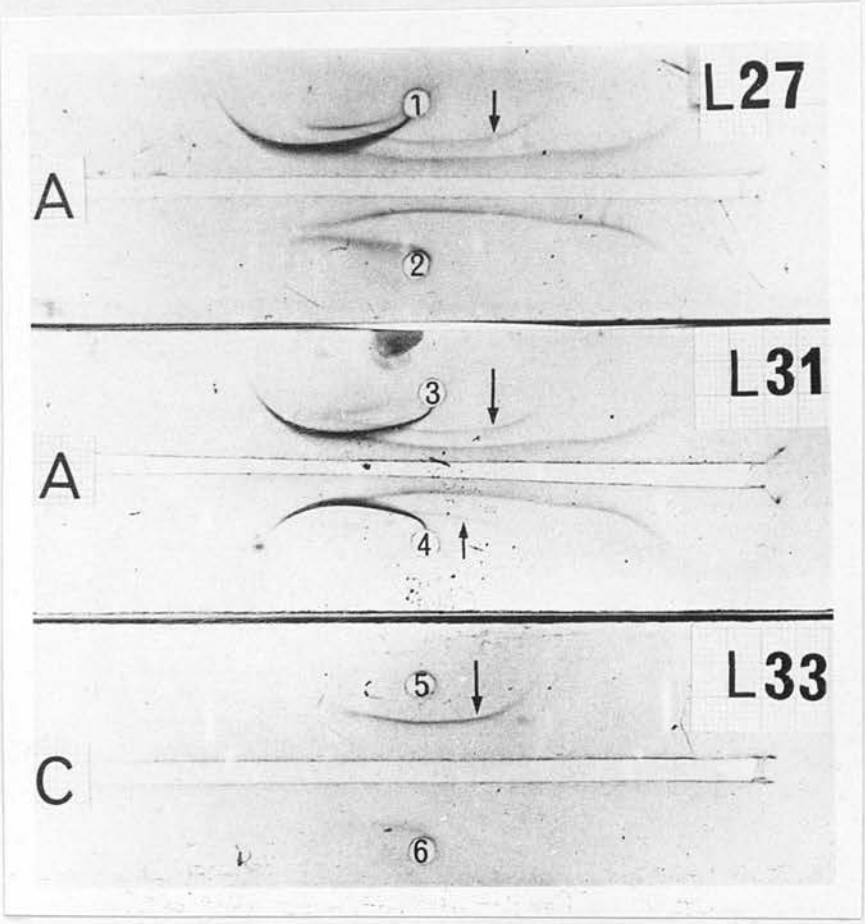
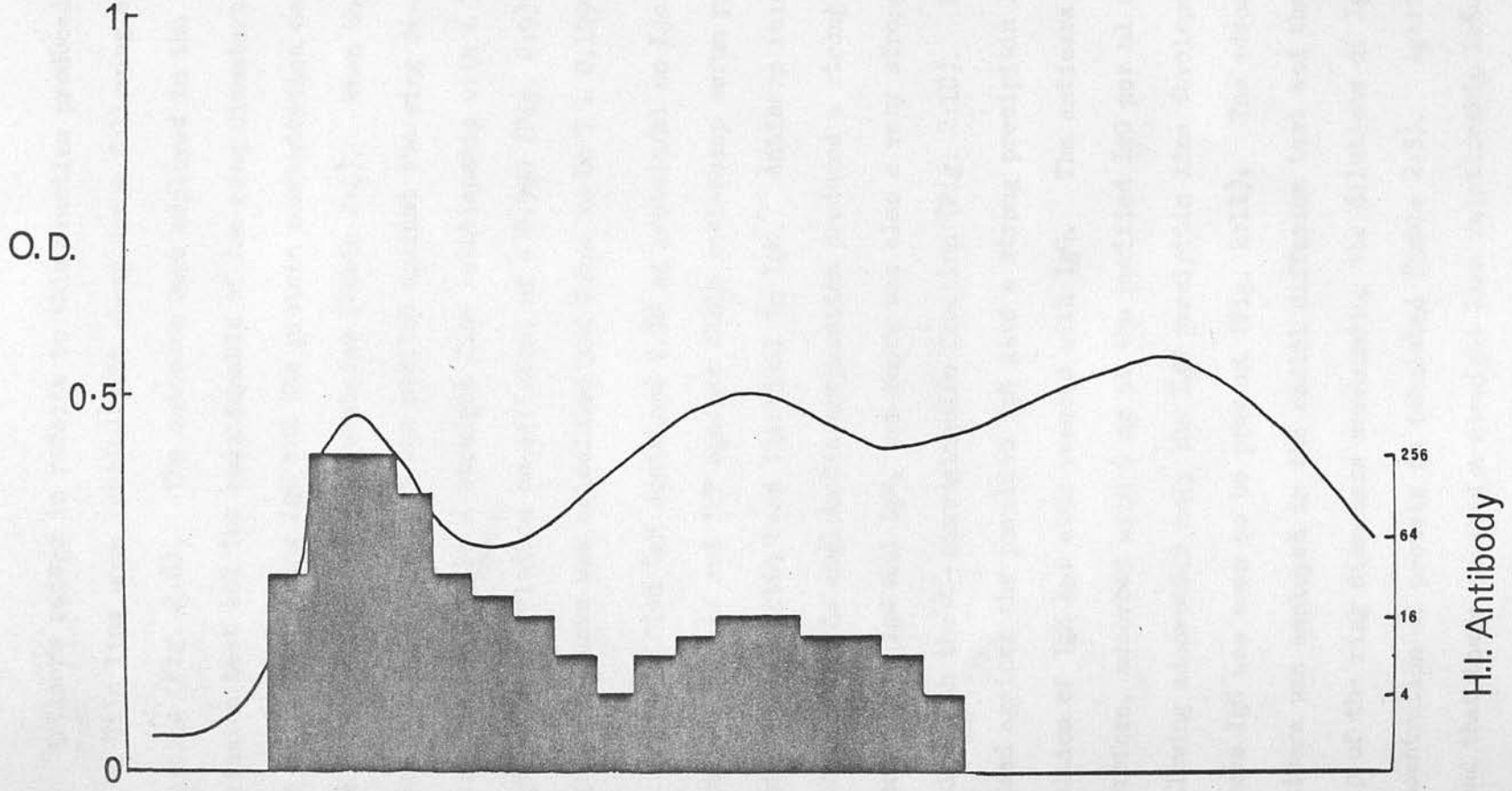


Fig. 2:6. Origin 1 charged with whole sheep serum, 2 and 6 charged with serum treated at 64.5° C for 30 mins., 3 charged with serum treated at 56° C for 30 mins., 4 charged with serum treated at 62.5° C for 30 mins., 5 charged with serum treated at 60° C for 30 mins.

Fig. 2:7. Distribution of haemagglutination-inhibiting (HI) antibody following G-200 filtration of serum collected from a lamb with acute louping-ill virus infection.


1
O.D.
0·5
0
256
64
16
4
H.I. Antibody

Antisera raised in rabbits to this fraction produced a single precipitin line when reacted with sheep serum following electrophoresis (Fig. 2:8). The antisera were employed in the radial diffusion test and the relationship of the ring diameters around the wells containing IgG and the protein concentration contained in the dilutions of IgG are tabulated (Table 2:4). When the $\log_{10}$ concentration of protein was plotted against the ring diameter there was seen to be a straight line relationship with a linear regression correlation co-efficient of + 0.990 (Fig. 2:9). The regression curve was calculated and found to be $Y = 0.1999\ X - 0.2955$.

The purified IgM contained 2.58 mg protein/ml on Kjeldahl protein analysis and IEP against whole anti-sheep serum produced a single precipitin band identical to IgM. Antisera raised in rabbits with the euglobulin preparation produced a strong precipitin line to both IgG and IgM, but there was also a very strong precipitation with the α-macroglobulin fraction (Fig. 2:10). The antisera raised against the purified IgM gave a strong precipitin line in the position of IgM but also reacted with IgG. The antisera were, therefore, adsorbed with 6 mg of the purified IgG per ml of serum. Following adsorption only the IgM precipitin line developed and excess IgG was seen to be present (Fig. 2:11). The adsorbed antisera was employed in the radial diffusion test and the relationship of the ring diameters surrounding the dilutions of IgM to the concentration of protein is tabulated (Table 2:5). Again it was found that there existed a straight line relationship between the ring diameters and the $\log_{10}$ concentration of protein with a linear

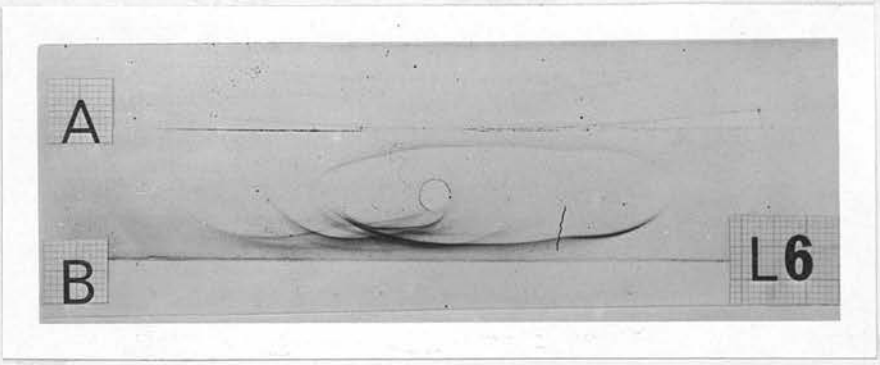
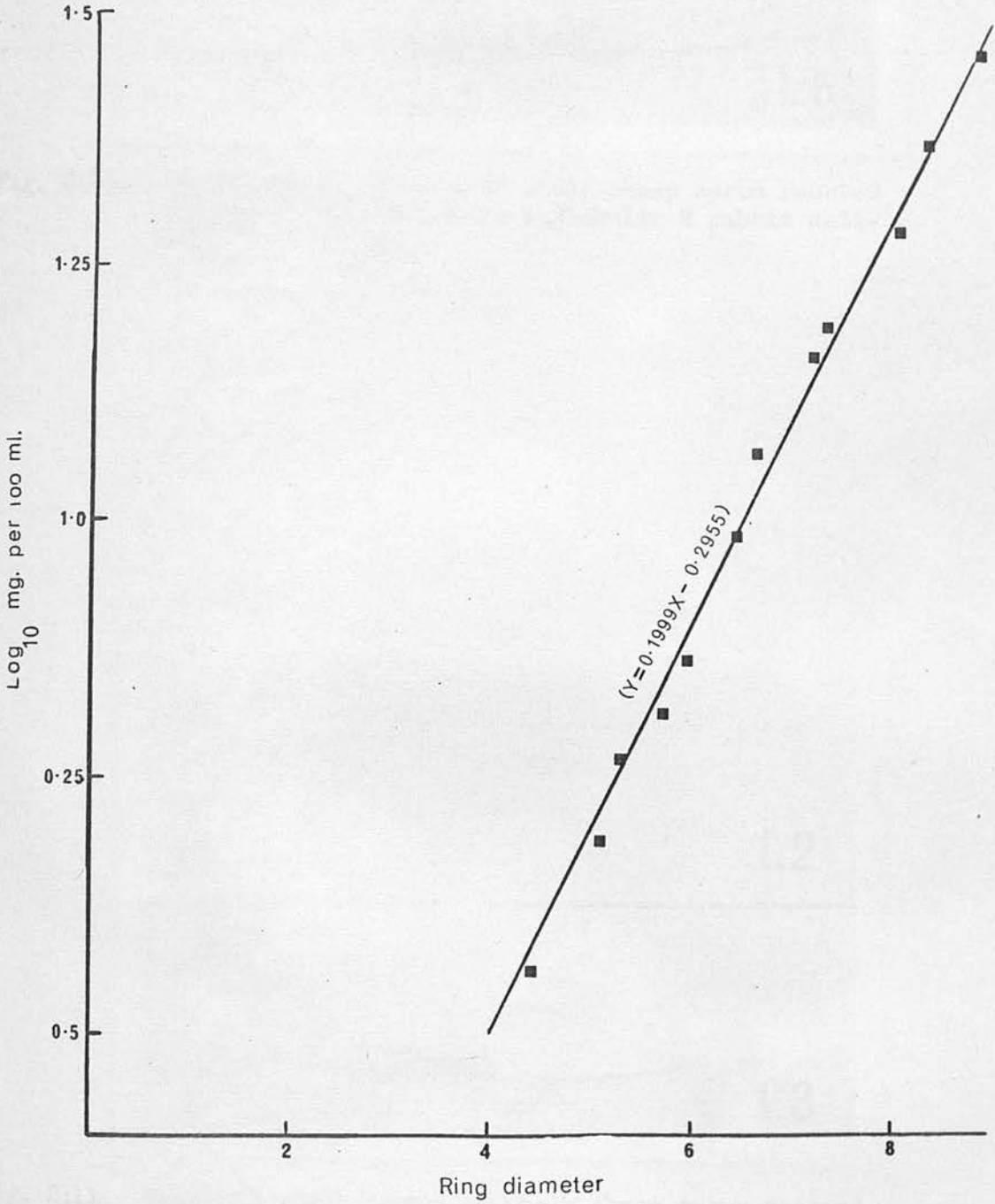


Fig. 2:8. Immunoelectrophoresis of whole sheep serum reacted with (A) rabbit anti-sheep IgG and (B) rabbit anti-whole sheep serum.

Table 2:4. The relationship of the ring diameter to protein concentration of IgG tested by the single ring diffusion technique.

Dilution	mg/100 ml	$\log_{10}$	Ring diameter
640	3.663	0.5639	4.45
480	4.884	0.6888	5.15
400	5.860	0.7679	5.24
360	6.511	0.8137	5.70
320	7.325	0.8648	5.93
240	9.768	0.9898	6.20
200	11.72	1.0690	6.60
160	14.65	1.1669	7.15
150	15.63	1.1939	7.31
120	19.53	1.2907	8.03
100	23.44	1.3699	7.75
80	29.30	1.4669	8.80

Fig. 2:9. The relationship of the $\log_{10}$ concentration of IgG to the precipitin ring diameter.


1·5
1·25
1·0
0·25
0·5
Log10 mg. per 100 ml.
(Y=0·1999X - 0·2955)
2
4
6
8
Ring diameter

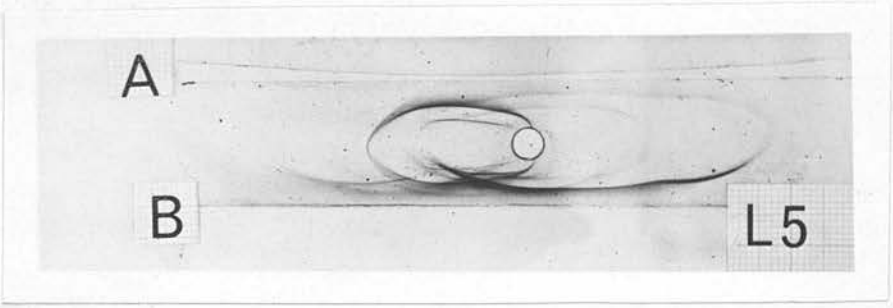


Fig. 2:10. Immunoelectrophoresis of whole sheep serum reacted with A rabbit anti-sheep euglobulin B rabbit anti-whole sheep serum.

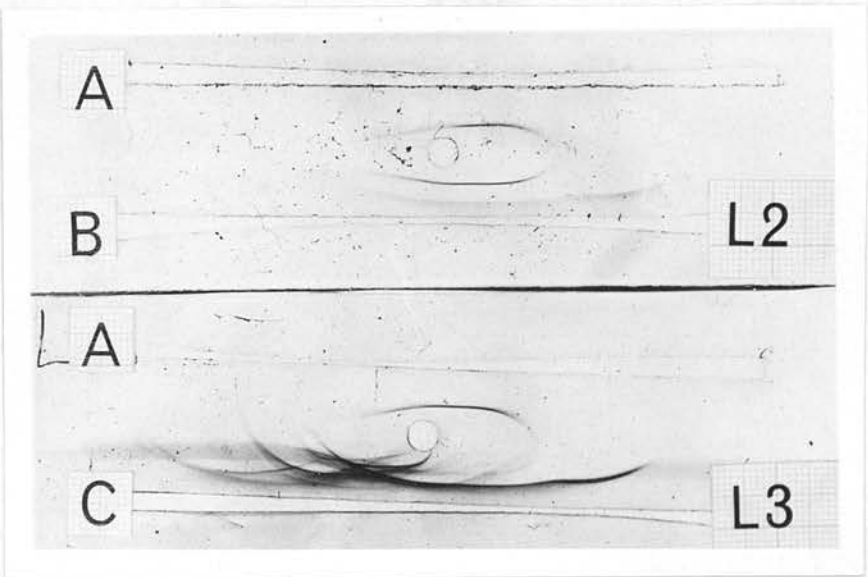
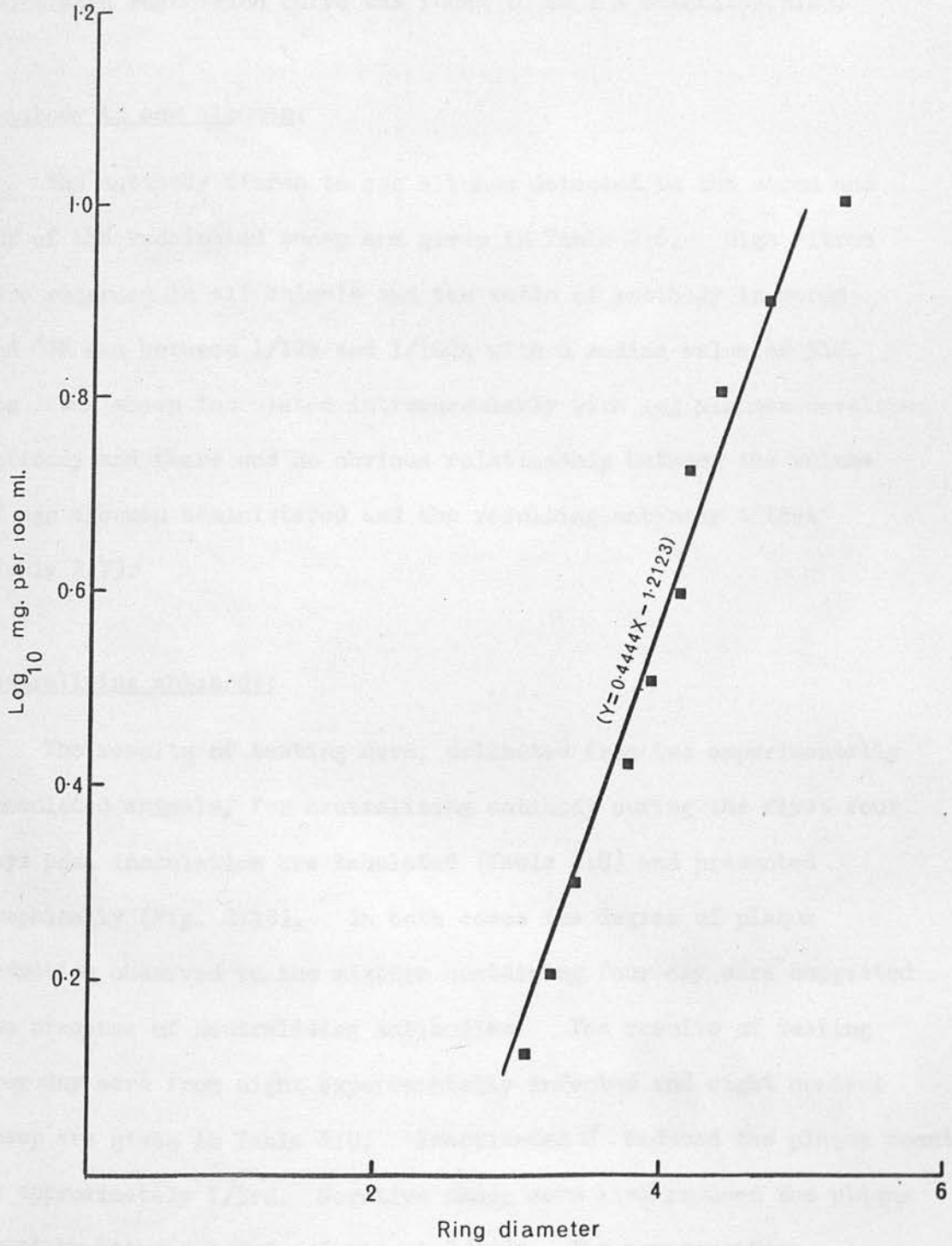


Fig. 2:11. Immunoelectrophoresis of whole sheep serum reacted with A rabbit anti-sheep IgM adsorbed with sheep IgG, B rabbit anti-sheep IgM before adsorption and C rabbit anti-whole sheep serum.

Table 2:5. The relationship of the ring diameter to the protein concentration of IgM tested by the single ring diffusion technique.

Dilution	mg/100 ml	$\log_{10}$	Ring diameter
220	1.17	0.0682	Trace
192	1.34	0.1271	3.07
160	1.62	0.2095	3.33
128	2.02	0.3054	3.38
96	2.68	0.4281	3.75
80	3.23	0.5092	3.9
64	4.03	0.6053	4.12
48	5.40	0.7324	4.15
40	6.45	0.8096	4.35
32	8.06	0.9063	4.71
24	10.79	1.0331	5.27

Fig. 2:12. The relationship of the $\log_{10}$ concentration of IgM to the precipitin ring diameter.


1·2
1·0
0·8
0·6
0·4
0·2
Log10 mg. per 100 ml.
(Y=0·4444X − 1·2123)
2
4
6
Ring diameter

regression correlation co-efficient of + 0.980 (Fig. 2:12). The calculated regression curve was found to be Y = 0.4444X-1.2123.

Antibody to egg albumen:

The antibody titres to egg albumen detected in the serum and CSF of the vaccinated sheep are given in Table 2:6. High titres were recorded in all animals and the ratio of antibody in serum and CSF was between 1/128 and 1/1024 with a median value of 512. The seven sheep inoculated intramuscularly with egg albumen developed antibody and there was no obvious relationship between the volume of egg albumen administered and the resulting antibody titres (Table 2:7).

Neutralizing antibody:

The results of testing sera, collected from two experimentally inoculated animals, for neutralizing antibody during the first four days post inoculation are tabulated (Table 2:8) and presented graphically (Fig. 2:13). In both cases the degree of plaque reduction observed in the mixture containing four day sera suggested the presence of neutralizing antibodies. The results of testing four day sera from eight experimentally infected and eight control sheep are given in Table 2:9. Inactivated C' reduced the plaque count by approximately 1/3rd. Negative sheep sera also reduced the plaque count by between 1/3rd and almost 2/3rds. The non-specific neutralizing activity of sheep and guinea pig sera appeared to be

Table 2:6. Reciprocal passive haemagglutination antibody titres in sheep serum and CSF to egg albumen following inoculation with a vaccine prepared from infected egg embryos.

Sheep No.	Serum	CSF	Ratio
9K00	32,768	32	1.024
9K05	4,096	4	1,024
9K06	8,192	32	256
9K13	16,384	64	256
9K16	8,192	32	256
9K19	65,536	64	1,024
9K22	131,072	256	512
9K24	131,072	1,024	128
9K30	8,192	16	512

Table 2:7. Antibody response of sheep to egg albumen measured by the Passive Haemagglutination test, expressed as reciprocal values.

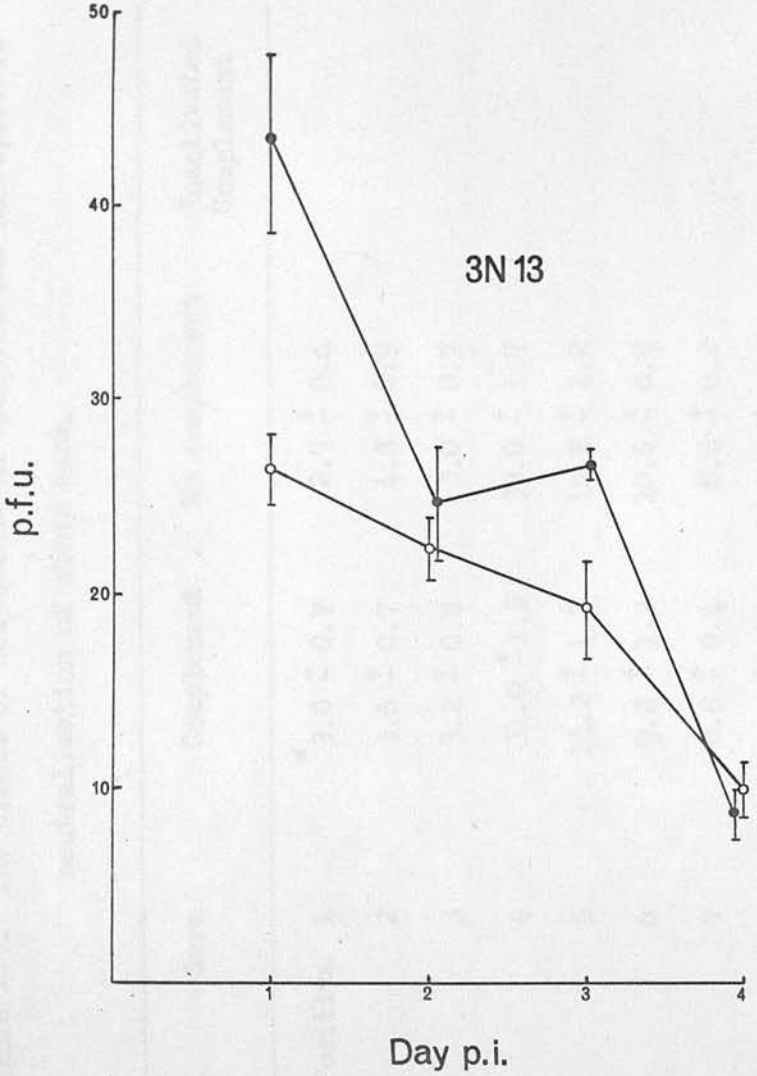
Animal	Volume Inoculated	Day post-inoculation 0	5	10	15	20
2K63	10 ml	< 10	20	320	320	320
2K65	5 ml	< 10	20	40	80	80
2K71	2 ml	< 10	160	160	160	40
8K02	1 ml	< 10	40	80	80	160
8K04	1 ml	< 10	40	160	160	160
8K06	1 ml	< 10	160	1280	640	640
8K07	1 ml	< 10	20	640	1280	640

Table 2:8. Plaque reduction by sheep serum following inoculation with louping-ill virus.

Day post-inoculation	Complement	Infected Sheep No. 3N13	3N18	Control Sheep 3N15
1	Present	28.2* ± 1.8	24.6 ± 2.6	24.0 ± 1.8
	None	43.4 ± 4.8	29.8 ± 2.4	34.4 ± 2.9
2	Present	23.0 ± 1.6	24.0 ± 2.6	
	None	24.8 ± 2.9	21.4 ± 0.5	
3	Present	19.3 ± 2.5	22.2 ± 1.8	
	None	26.8 ± 0.75	23.0 ± 3.4	
4	Present	10.0 ± 1.1	9.5 ± 1.4	
	None	8.8 ± 1.3	13.0 ± 1.2	

* Mean number of plaque forming units (± SE).

Fig. 2:13. The mean ($\pm$ SE) plaque count following incubation with sheep serum collected on the first 4 days following challenge with louping-ill virus, with the addition of complement (—o—) and without (—●—).


3N 13
p.f.u.
50
40
30
20
10
1
2
3
4
Day p.i.

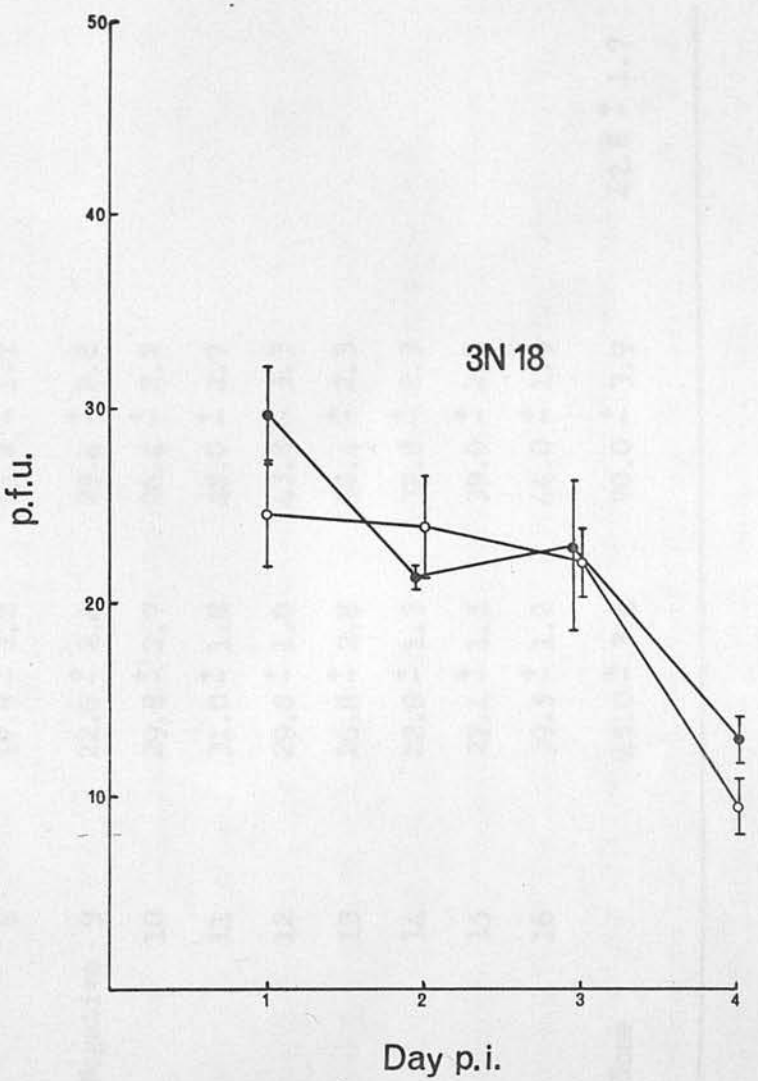

3N 18
p.f.u.
50
40
30
20
10
1
2
3
4
Day p.i.

Table 2:9. The effect of complement on specific and non-specific neutralization of sheep sera.

Sera		Complement	No complement	Inactivated Complement
Positive	1	*3.0 ± 0.7	2.9 ± 0.4	
	2	3.6 ± 0.7	4.8 ± 0.9	
	3	3.2 ± 0.8	3.0 ± 0.7	
	4	31.0 ± 1.5	33.0 ± 1.7	
	5	14.2 ± 1.2	14.8 ± 1.2	
	6	9.8 ± 1.3	10.6 ± 0.9	
	7	0.8 ± 0.4	0.8 ± 0.2	
	8	17.8 ± 2.2	15.2 ± 1.7	
Negative	9	22.6 ± 2.4	29.4 ± 2.8	
	10	29.8 ± 2.7	26.4 ± 2.2	
	11	31.0 ± 1.8	48.0 ± 2.7	
	12	29.8 ± 1.0	43.8 ± 3.3	
	13	26.8 ± 2.8	38.4 ± 2.3	
	14	22.8 ± 1.3	32.8 ± 2.3	
	15	27.4 ± 1.5	39.0 ± 2.0	
	16	39.5 ± 1.2	44.0 ± 1.9	
None		45.0 ± 3.4	70.0 ± 3.9	47.8 ± 1.7

*Mean plaque count ± SE.

generally additive in that the addition of C′ or inactivated C′ tended to reduce the plaque count in the serum virus mixtures by an additional 1/3rd. C′ did not exert a consistent effect in the positive serum virus mixtures and in no case was enhanced specific neutralization suggested.

Interference:

The mean plaque count (± SE) of mixtures of virus and medium, normal mouse brain or inactivated virus were 25.3 (± 1.7), 21.6 (± 2.8) and 22.4 (± 1.6) respectively. The inactivated virus contained $10^{6.7}$ pfu. per 0.2 ml prior to inactivation; there was thus no evidence of interference due to inactivated virus.

DISCUSSION

Both kaolin and acetone appeared equally satisfactory for removing NSHI from sheep serum. Due to the low concentration of protein in CSF neither the acetone nor the kaolin method of extracting NSHI were satisfactory (Webb et al, 1968a). These authors found the addition of 0.5% BA rendered the acetone method satisfactory which was confirmed in the tests described here. However, as the highest level of NSHI in CSF was found to be 1/2.5 and as the addition of BA caused panagglutination in the PHA test, extraction of CSF prior to testing for antibody was omitted in this

study.

The results presented confirmed those of Jonas (1969) that heat treatment and 2-ME can effectively remove antibody activity associated with the IgM class of antibody from sheep serum. Both heat and 2-ME treatments reduced the HI activity in sera from clinically affected animals and animals that had been infected recently with louping-ill virus. Activity in serum from a hyper-immunised sheep was unaffected by either treatment. In acute phase serum, but not in hyperimmune serum, some of the antibody activity would be expected to be associated with IgM if sheep react to infection with louping-ill virus in a similar fashion to that observed in calves infected with other togaviruses (Sanderson, 1968a,b). Thus treatment with 2-ME or heat appeared to specifically reduce the HI antibody titre associated with IgM.

This was confirmed by both IEP and Sephadex G-200 filtration. No IgM precipitin line could be detected by IEP of serum treated with 2 M 2-ME or heated at 64.5° C, while the IgG precipitin line remained unaltered in such samples. The HI activity of a serum collected from a lamb with acute louping-ill virus infection was distributed over the first two exclusion peaks following Sephadex G-200 filtration. However, activity was only found in the eluates from the 2nd peaks when samples of the same serum were treated with heat or 2-ME prior to fractionation.

The data therefore indicated that either method of treatment was of value in assessing the class of antibody causing HI; the

titre in untreated samples being due to the activity of IgG and IgM while that remaining after treatment being due to IgG alone. The reduction involved was therefore a measure of the activity due to IgM.

The tests for quantitating both IgM and IgG appeared satisfactory and the linear relationship of the precipitin ring diameters with the $\log_{10}$ concentration of protein was in agreement with the results of Fahey and McKelvey (1965). The results reported here for the purified IgM and IgG were derived at the same time as the test samples reported later. The concentration of IgM and IgG in the test specimens could, therefore, be derived from the respective calculated regression curve. This was found more convenient than the comparison of ring diameters of test samples against the graphic curve obtained with a standard immunoglobulin as recommended previously (Fahey and McKelvey, 1965).

High levels of antibody to egg albumen were found in the serum of sheep inoculated with a vaccine prepared in egg embryos. The ratio of antibody in serum and CSF had a median value of 512, which was of the same order as that recorded by Clarke, Dane and Dick (1965) for antibody to poliovirus in man. It was, therefore, concluded that antibody to egg albumen as measured by the PHA provided a useful serum antibody marker. All the sheep inoculated intramuscularly with egg albumen developed antibody.

In the sheep tested, specific neutralizing activity was first detected in four day sera. In addition variable levels of natural

inhibitors were found in sheep serum. There was no evidence that C′ enhanced the specific or non-specific activity.

Although fresh guinea pig serum has been found to potentiate neutralization of some togaviruses the effect has not been marked, nor has it invariably been detected (Westaway, 1965; Iwasaki and Ogura, 1968; Spector and Tauraso, 1969).

Iwasaki and Ogura (1968) found C′-potentiated neutralization of four togaviruses was only detected when the virus used in the production of antiserum and for detecting antibody was grown in the same cell system. They did not detect C′ -potentiated neutralization when the virus used for antibody production was grown in different cell systems to that used for detecting antibody. Thus they suggested that the potentiation of neutralization by C′ may at least in part be due to antibody directed against antigenic determinants of host cell origin present in the virus envelope.

In the present experiment the antisera were selected from infected sheep and the virus employed was prepared from suckling mouse brain. The suggestion of Iwasaki and Ogura would thus explain the failure to detect potentiated neutralization with C′ in this work.

The sera tested for neutralizing antibody were collected from sheep during the acute phase of infection at which time viraemia was present. Sera heated at 56° C for 30 minutes were therefore anticipated to contain varying quantities of inactivated virus. There was no evidence that inactivated virus exerted an effect on

the plaque count when $10^{6.4}$ pfu per 0.2 ml of inactivated virus was present. It was, therefore, assumed that the levels of virus present in serum would not cause a reduction in the plaque count due to interference. This is in accordance with the results found with other togaviruses in chick embryo cell cultures (Vilček, 1963) and pig kidney cells (David-West and Porterfield, 1974) in which no interference could be detected due to inactivated virus even when up to 100 ID_{50}/cell was added.

It was evident from these limited experiments that sheep sera contained natural inhibitors at various levels. In order to detect the development of specific neutralizing activity, in sera from individual animals allowance had to be made for the presence of such inhibitors, hence the following scheme was devised. The level of natural inhibitors (N) present in serum was expressed as that fraction of virus remaining following incubation with pre-inoculation serum. Hence $N = \frac{Po}{Co}$, where Po in the observed plaque count following incubation with pre-inoculation serum and Co is the observed plaque count in the control cultures on the day of the test.

To determine the level of specific neutralizing activity in a serum sample tested on day y, the observed plaque count following incubation with that serum (Py) was corrected for natural inhibitors present in the pre-inoculation serum of the individual concerned. Before this could be done, it was necessary to adjust Py according to the control plaque count (Cy), for the day when Py was determined

to correspond to the control count (Co) on the day when the pre-inoculation serum was tested. This may be expressed as follows: the adjusted plaque count $(\overline{Py}) = \frac{Co}{Cy} \times Py$. The adjusted fraction is due to both specific neutralization (S) and the activity of N, hence $\frac{\overline{Py}}{Co} = S + N$. Specific antibody can however only act upon the residual fraction of virus left following reduction by N. In order to compare the specific neutralizing capacity of sera, independently of N it is required to express this value as $\frac{S}{N}$. Thus, to calculate $\frac{S}{N} : \frac{\overline{Py}}{Co} \times \frac{1}{N} = \frac{S + N}{N}$

$$= \frac{S}{N} + 1$$

$$\frac{S}{N} = \left(\frac{\overline{Py}}{Co} \times \frac{1}{N}\right) - 1$$

To further explain the method used for detecting neutralizing antibody the complete results from the sera collected from one animal are presented in Appendix 2:1.

The validity of this test depended on the assumption that the level of natural inhibitors in serum collected sequentially from an individual animal would remain relatively constant. That this was the case emerged from processing sera from control animals of experiments to be described in the next chapter. The N values for sera from control sheep collected before inoculation with normal mouse brain $(\frac{Po}{Co})$ and the $\frac{S}{N}$ values 3 and 5 days later are presented in Appendix 2:2. The level of N in serum collected pre-inoculation varied from 0.5095 to 0.7942. The $\frac{S}{N}$ values of sera collected subsequently generally were close to zero. Thus it is clear that

the levels of N of an individual animal tend to remain constant at least for a period of one week.

The reproducibility of the test was investigated when processing the sera from experimentally infected sheep. A number of sera were tested on two occasions and the $\frac{S}{N}$ values calculated (Appendix 2:3). There was excellent agreement between the results of the two tests ($P > 0.70$), the largest difference being 0.0951.

The plaque reduction test, therefore, appeared a satisfactory method for the detection of serum neutralizing antibody in sheep serum following experimental infection.

CHAPTER 3

EXPERIMENTAL LOUPING-ILL IN SHEEP

INTRODUCTION

Following i.c. inoculation of sheep with louping-ill virus the outcome was invariably fatal (Greig et al, 1931). However, following s.c. inoculation only a proportion of animals developed clinical disease (Poole et al, 1930; Gordon et al, 1932a; MacLeod and Gordon, 1932; Edwards, 1947). An experiment was, therefore, performed to determine if comparison of the nature of the viraemia and antibody response in i.c.- and s.c.-challenged animals might provide evidence as to the reasons for this variability in response following s.c. challenge. In a second experiment a critical comparison was made of the immune response and the viraemia that developed in sheep that became severely affected and in those that did not, following s.c. inoculation.

Primary isolates of togaviruses have been shown to consist of sub-populations possessing distinct characteristics (Henderson, 1964; Libíková, Řeháček, Mayer, Kožuch and Ernek, 1964). Passage of such isolates in laboratory systems may selectively favour certain sub-populations (Mayer, 1963; Mayer and Rajčáni, 1967, 1968; Mayer and Kožuck, 1969; Henderson, Shah and Wallis 1965; Henderson, Levine, Karabatsos and Stim, 1967; Henderson, Levine, Stim and Karabatsos, 1967). Studies of the pathogenicities

of togavirus infections should, therefore, be performed using isolates that have undergone few laboratory passages if the results are to be related to the naturally occurring disease. For this reason two strains of louping-ill virus isolated from the brains of clinically affected sheep were used following only one mouse passage in the first challenge experiment. In the second experiment only one of these strains was used following further passage once in lamb blood and once in sucking mouse brain.

In the first experiment detection and titration of virus was performed by i.c. inoculation of mice while in the second experiment virus assay was performed using the plaque method.

PART I - The response following intracerebral and subcutaneous inoculation.

MATERIALS AND METHODS

Twenty, nine-month-old Scottish Blackface and Blackface-cross sheep were purchased from a tick-free environment. Groups of four of these were inoculated i.c. with 1.0 ml into the right cerebral hemisphere or s.c. with 10.0 ml into the medial aspect of the right thigh. Thus eight sheep were inoculated with each virus inoculum. An additional two sheep were inoculated i.c. with control material and two were inoculated s.c. Each virus inoculum was titrated by i.c. inoculation of mice, immediately after injecting the experimental sheep. The sheep inoculated i.c. with SB/526 and SB/527 received $10^{6.2}$ and $10^{5.9}$ mouse i.c.

ID_{50} respectively and animals inoculated s.c. received 10 times these amounts. Following inoculation animals were inspected at frequent intervals and those that developed severe signs of neurological dysfunction were killed in extremis and survivors were killed 20 days post-inoculation.

Blood samples (8.0 ml) were collected immediately prior to inoculation and at 5, 10, 15 and 20 days thereafter. In addition, terminal samples were taken from all moribund animals. Blood was allowed to clot at room temperature and kept overnight at $+4^{\circ}$ C. Serum was pipetted off, centrifuged at 1,000g for 15 minutes at room temperature and the supernatant fluid stored at -70° C. HI antibody was tested for as described earlier using both 2-ME and heat treatment to assess the relative activity due to IgM.

For virological examination sheep were bled from the jugular vein using a 19 gauge needle and 1 ml syringe, at 12 hour intervals from the 6th hour post inoculation for ten days. Each sample was immediately diluted 1:5 in chilled HSS before clotting occurred. Two aliquots of each of the specimens were stored at -70° C in screw cap bottles until all specimens had been collected.

One aliquot from each pair was thawed quickly at 37° C, centrifuged at 1,200g and the supernatant fluid screened for virus in a group of five mice. The duplicate aliquots found positive following this initial screening, were thawed,

centrifuged and diluted in ten-fold steps in HSS for inoculation of mice. Initially all samples were diluted to 10^{-6} but as none of the sheep tested in the early stages of the work had titres greater than $10^{2.0}$ samples were normally thereafter only diluted to 10^{-4}.

RESULTS

All the animals that were inoculated i.c. with virus developed signs of severe neurological dysfunction four to six days after inoculation. Typically there was a progression of symptoms from slight ataxia to complete flaccid paralysis over a period of six to 18 hours. Animals were killed in extremis 117 to 153 hours after inoculation (Table 3:1). There was no difference in the incubation time ($P < 0.5$) between sheep given SB/526 and SB/527.

One sheep (3K45) that had been inoculated s.c. with SB/526 was killed when moribund at 217 hours post infection. The other seven that had been given virus s.c. survived until the experiment was terminated. However, neurological symptoms did develop in three of them; 3K41 developed a permanent non-progressive posterior paresis from day 12 and 3K28 was ataxic on days eight to ten, as was 3K43 on days 11 and 12. Thus three of four sheep inoculated with SB/526 and one of four inoculated with SB/527 reacted clinically following s.c. inoculation.

The controls remained healthy throughout the period of

Table 3:1. The viraemia* detected in sheep following intracerebral inoculation with one of two isolates of louping-ill virus.

Virus isolate		SB/527				SB/526			
Sheep No.		3K30	3K36	3K38	3K35	3K34	3K32	3K37	3K33
Hours after inoculation	6	N	N	N	N	N	T	N	
	18	N	N	T	N	N	N	N	N
	30	N	N	N	N	T	T	T	N
	42	N	N	N	N	T	1.5	1.5	T
	54	N	T	T	N	0.5	2.0	2.4	T
	66	T	T	T	N	0.6	3.0	3.6	T
	78	T	1.4	2.0	N	1.8	1.5	>4.0	1.2
	90	N	2.4	2.4	N	1.0	1.6	>4.0	2.3
	102	N	2.4	T	N	T	1.2	>4.0	1.5
	114	N	T	N	N	N	N	3.0	1.7
	126	(117)	N	N	N	(117)	N	1.8	T
	138		(121)	(128)	N		(127)	T	N
	150				(139)			N	N
								(152)	(153)

*Expressed as $\log_{10} ID_{50}$/0.03 ml of original blood.

T = Trace N = No virus isolated ( ) = Time at which moribund animals were killed.

Table 3:2. The viraemia* detected in sheep following subcutaneous inoculation with one of two isolates of louping-ill virus.

Virus isolate		SB/527				SB/526			
Animal No.		3K43	3K44	3K42	3K39	3K45/	3K41	3K29	3K28
	6	N	N	N	N	N	N	N	N
Hours	18	N	N	N	N	T	N	N	T
	30	T	N	N	N	1.6	N	T	T
after	42	T	N	T	N	1.6	N	0.6	T
inocula-	54	T	T	0.6	N	2.0	T	1.5	T
tion	66	1.5	T	T	T	2.7	T	T	1.8
	78	2.2	T	2.5	T	2.0	T	1.8	2.4
	90	1.5	T	1.6	T	1.5	T	0.8	1.5
	102	T	T	2.2	N	T	T	0.6	T
	114	T	N	1.6	N	N	T	T	T
	126	T	T	T	N	N	N	N	N
	138	N	N	T	N	N	N	N	N
	150	N	N	T	N	N	N	N	N
	162	N	N	T	N	N	N	N	N
	174	N	N	N	N	N	N	N	N

*Expressed as $\log_{10}ID_{50}$/0.03 ml of original blood.

T = Trace N = No virus isolated.

/ = Sheep 3K45 was killed *in extremis* at 171 hours after inoculation. The other animals survived.

observation.

The duration and magnitude of the viraemias are shown in Table 3:1 and 3:2. Concentrations of virus in excess of 10^3 mouse ID_{50}/ml of blood were recovered on at least one occasion from 11 animals. Virus levels lower than this were detected in four others, but were not detected at any time in one of the sheep inoculated i.c. with SB/527. Virus was not recovered from any of the controls.

The viraemia in sheep inoculated i.c. with SB/526 tended to be greater than in sheep given SB/527 i.c., though all animals succumbed and the incubation period in both groups was of the same order. In all cases the viraemia terminated prior to the appearance of clinical signs. There was a marked variation in the duration and magnitude of the viraemia detected in animals inoculated s.c., but there was no obvious difference between the two isolates. The animal that succumbed (4K45) did, however, develop the most intense viraemia.

Serum HI antibody was demonstrated in all animals inoculated s.c. with virus, but was not found in any of the animals inoculated i.c. or in the animals that received control material. The extent of the reduction in activity following the treatment of serum by heat and 2-ME were similar (Table 3:3). Thus, either method appeared satisfactory for assessing the relative HI activity associated with the IgG and IgM classes of immunoglobulin, in large numbers of samples.

Table 3:3. Haemagglutination-Inhibiting antibody to louping-ill virus in sheep serum following subcutaneous inoculation with one of two isolates.

Virus isolate		SB/527				SB/526			
Sheep No.		3K43	3K44	3K42	3K39	3K45	3K41	3K29	3K28
PBS+	5/	<10	<10	<10	20	80	20	10	<10
	10	>2560	>640	5120	1280	2560	>640	>1280	>2560
	15	>5120	>320	5120	1280	(7)	1280	1280	2560
	20	2560	640	2560	640		2560	640	2560
Heat	5	<10	<10	<10	<10	<10	<10	<10	<10
	10	>20	40	80	40	20	20	>160	NE
	15	2560	>80	2560	160		320	40	NE
	20	2560	>160	1280	160		640	80	320
2-ME	5	<10	<10	<10	<10	<10	<10	<10	<10
	10	>160	20	80	20	80	10	40	80
	15	1280	160	2560	80		160	>160	640
	20	>1280	>160	1280	80		1280	>80	320

Titres are expressed as reciprocals.

/ = Days after inoculation.

( ) = Day of terminal sample from moribund animal.

NE = Not examined.

+ = Treated with PBS as a control.

The predominant HI activity in sera collected early in the course of infection was due to IgM, but was still present on day 20 though contributing less to the total activity. Antibody was detected on day five in three of the four sheep inoculated s.c. with SB/526, but was only present in one of the four inoculated with SB/527. All animals were positive on day ten, when the activity in untreated sera was at a maximum in five of the seven surviving sheep. Maximum activity in the IgG class of antibody was recorded on day 15 in six sheep and on day 20 in one.

PART 2 - Comparison of the response in "susceptible" and "resistant" sheep.

MATERIALS AND METHODS

Forty-three six-to-nine-month-old sheep purchased from a tick-free environment and shown to be free of HI antibody to louping-ill virus were each inoculated intramuscularly with 1 ml of a 20% solution of egg albumen. Twenty-two days later 35 of the sheep were inoculated subcutaneously with $10^{7.4}$ p.f.u. of the SB/526 isolate of louping-ill virus, and eight were given control material. Sheep were bled prior to inoculation and every 24 hours afterwards for heparinized[1] (4.0 i.u. per ml), whole blood and serum using evacuated tubes. Thus a total of 30.0 ml of blood was collected daily from each sheep for the first ten

[1] Pullarin, Evans Medical Ltd., Liverpool, England.

days post-inoculation. Thereafter sheep were bled periodically for serum only.

Animals were inspected at frequent intervals and all sheep that became moribund were sacrificed, animals that survived were killed from day 28 onwards. Prior to decapitation blood for serum separation and CSF obtained from the cisterna magna were collected. Any samples of CSF in which red cells could be detected on visual inspection were discarded. Following decapitation pieces of cerebrum, cerebellum and spinal cord were collected and stored at -70° C for virus isolation attempts.

Plasma was separated from the heparinized blood by centrifugation at 1500 g at + 4° C for 20 minutes. Each of the freshly separated plasmas was inoculated on to four or five plates and two aliquots of 0.5 ml of the remaining sample were then stored in glass sealed ampoules at - 70° C. If a confluent cytopathic effect developed a duplicate sample of the frozen plasma was thawed and diluted in growth medium for virus assay. Plaques were counted and the mean number of p.f.u. per 0.2 ml of plasma calculated. Samples of nervous tissue were thawed and homogenised as 10% suspensions in growth medium using a Teflon grinder. The homogenates were clarified at 1500 g for 15 minutes at + 4° C and the concentration of virus in the supernatant fluid determined by plaque assay and expressed as p.f.u. per 0.2 gm of tissue. Undiluted CSF was also tested for virus.

HI antibody to louping-ill virus and PHA antibody to egg

albumen were determined in serum and CSF samples while neutralizing activity was only assayed in serum samples. Assessment of the HI activity due to IgM was made of serum and CSF samples using 2-ME. The relative HI activity in the IgG and IgM classes of antibody of three samples were also assessed using Sephadex G-200 filtration. Two ml aliquots of terminal serum were extracted with acetone and the final precipitates were reconstituted in 2.0 ml of tris-HCl. buffer. One ml volumes of these extracted sera and of the paired unextracted CSF were applied separately to a column and the HI activity determined in the first two exclusion peaks. Total levels of IgM and IgG in CSF were compared with serum collected terminally and total protein levels were estimated by Kjeldahl analysis.

RESULTS

Clinical response:

One animal died five days after infection and Pasteurella haemolytica was isolated from blood, liver, kidney and lung homogenates (Gilmour, personal communication) and the histological examination was consistent with a diagnosis of acute pasteurellosis (Angus, personal communication). One other animal was also found dead 28 days after infection, but the cause of death was not ascertained. Thus, the results from both these sheep were omitted from the analysis. The analysis was, therefore, confined to 33 infected animals, of which four died and 18 were killed with

severe signs of neurological dysfunction. These 22 animals which will be referred to subsequently as "susceptible" were moribund from six to 12 days after inoculation (Table 3:4). The symptoms observed in no way varied from those seen in the first experiment. Of the 11 other sheep that did not develop severe signs of encephalomyelitis two were chronically affected. These 11 will subsequently be referred to as "survivors".

The eight controls remained clinically normal throughout; no virus was isolated from their plasmas and they did not develop antibody to louping-ill virus.

Virus assay:

At 24 hours after inoculation virus was isolated from the plasmas of all except two of those inoculated with virus. One of the susceptible animals (3 NO4) did not develop detectable viraemia. The dynamics of viraemia in the 2 susceptible animals and 11 survivors that did circulate virus are summarized in Fig. 3:1. Maximum titres ranged from $10^{3.15}$ to $10^{7.51}$ and occurred on days three and four. The mean level of viraemia was slightly higher throughout in the susceptible animals; in addition maximum concentrations of virus recovered tended to be greater in those that succumbed ($P < 0.01$), in 10 cases exceeding 10^6 p.f.u. per 0.2 ml (Table 3:5). Titres of this level were not detected in any of the survivors. By day four, titres had declined in 41% of the susceptible animals and 73% of the survivors. On

Table 3:4. Cumulative mortality in sheep following subcutaneous inoculation with louping-ill virus.

Day post-inoculation	5	6	7	8	9	10	11	12
No. of animals moribund or dead	0	1	6	13	20	21	21	22
Percentage	0	6.7	18.2	39.4	60.6	63.6	63.6	66.7

Fig. 3:1. The mean ($\pm$ SE) level of viraemia following s.c. inoculation with louping-ill virus in 21 sheep that succumbed (●) and 11 that survived (○), expressed as $\log_{10}$ p.f.u. per 0.2 ml of plasma.

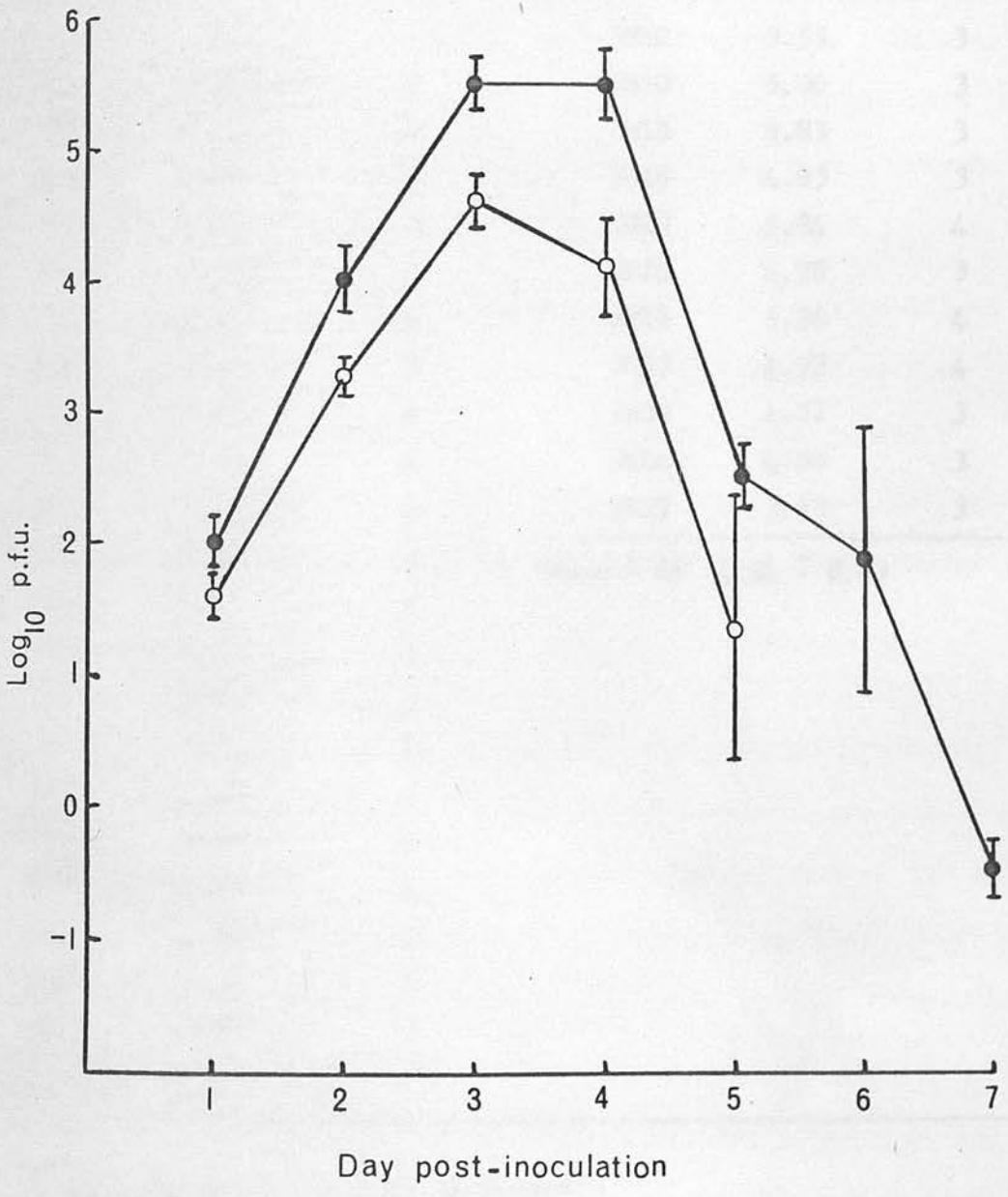

6
5
4
3
2
1
0
-1
Log10 p.f.u.
1
2
3
4
5
6
7
Day post-inoculation

Table 3:5. The maximum level of louping-ill virus detected in plasma of susceptible sheep and of sheep that survived.

Susceptible			Survived		
Sheep No.	Titre	Day of maximum viraemia	Sheep No.	Titre	Day of maximum viraemia
3N04	N	-	3N02	3.55	3
3N06	4.76+	4	3N10	5.00	3
3N07	7.33	4	3M13	5.83	3
3N08	4.98	3	3N18	4.83	3
3N09	4.85	3	3N20	5.84	4
3N11	5.68	3	3N25	4.78	3
3N12	7.00	4	3N26	5.56	4
3N14	7.51	3	3N33	4.73	4
3N16	5.69	4	3N37	4.72	3
3N17	5.79	4	3N44	4.86	3
3N21	7.11	4	3N47	3.53	3
3N22	6.26	3	Mean ± SE	4.84 ± 0.23	
3N24	6.62	4			
3N27	6.48	4			
3N29	5.87	3			
3N31	5.78	4			
3N32	5.18	4			
3N34	6.08	4			
3N38	6.15	4			
3N39	5.65	3			
3N41	3.15	3			
3N45	6.90	3			
Mean ± SE	5.95 ± 0.22				

+ Log_{10} p.f.u. per 0.2 ml of plasma.
N = No virus detected.

day five, levels of 10^2 p.f.u. per 0.2 ml were still present in 13 of the susceptible animals, but were found in only one of the survivors. Viraemia persisted until days six and seven in 81% and 33% of the remaining susceptible sheep, but was not found subsequent to day five in any of those that survived. On average, therefore, levels of viraemia were higher in susceptible than in surviving sheep, and terminated earlier in those that survived. The data are tabulated in full in Appendices 3:1 and 2.

Virus was isolated from the nervous system of all moribund animals (Table 3:6), but not from any of the controls or four survivors killed 28-35 days post-inoculation. The highest titres of virus recovered tended to be from the spinal cord, and virus was isolated from only one of the 12 CSF samples examined.

Serum antibody:

The decline in viraemia was associated with the appearance of circulating HI antibody and increasing levels of neutralizing activity in the serum. This is graphically represented for two animals, one of which succumbed (Fig. 3:2) and one which survived (Fig. 3:3). The development of HI antibodies in the susceptible and surviving sheep is summarized in Fig. 3:4.

In whole sheep serum HI antibody was first detected on day five in seven of the 11 survivors, but in only one of the 22 that succumbed. Thereafter titres rose rapidly until day eight in both groups, although higher mean levels were found consistently in

Table 3:6. Distribution of virus in the nervous tissue of sheep following subcutaneous inoculation.

Animal No.	Day	Cerebrum	Cerebellum	Spinal Cord	CSF
3N14	6	3.53+	4.90	3.43	NE
3N12	7	5.23	7.23	NE	NE
3N16	7	1.53	2.93	4.20	N
3N22	7	4.23	5.56	5.42	N
3N24	7	3.88	4.18	4.49	N
3N04	7	3.90	4.08	4.53	NE
3N07	8	4.08	5.60	6.51	1.59
3N27	8	3.76	4.72	5.48	NE
3N38	8	3.62	4.42	6.00	N
3N08	8	N	2.56	5.45	NE
3N29	8	2.45	4.04	5.81	N
3N32	8	3.20	4.36	5.76	NE
3N41	8	4.28	6.38	6.36	N
3N17	9	2.18	4.80	5.80	NE
3N45	9	N	2.42	5.04	NE
3N39	9	3.20	5.56	5.62	NE
3N34	9	N	2.36	3.53	N
3N21	9	NE	3.66	5.20	N
3N09	9	3.58	4.18	5.48	NE
3N11	9	2.34	3.38	5.64	N
3N06	10	0.30	2.08	3.20	N
3N31	12	N	NE	3.78	N

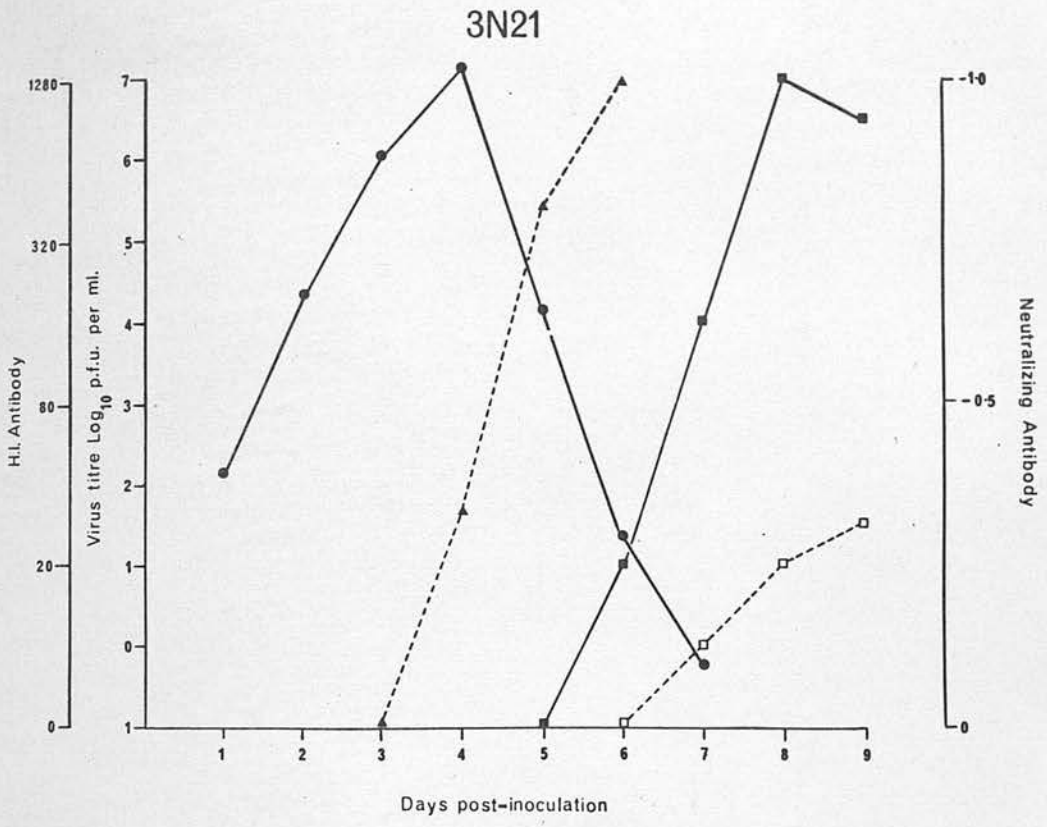
N = no virus detected.

NE = not examined

+ Log_{10} p.f.u.per 0.2 gm

Fig. 3:2. The development of viraemia (—●—), serum neutralizing antibody (- -▲- -) and HI antibody in whole serum (—■—) and following 2-ME treatment (- -□- -) in a sheep that died 9 days post-inoculation.

Fig. 3:3. The development of viraemia (—●—), serum neutralizing antibody (- -▲- -) and HI antibody in whole serum (—■—) and following 2-ME treatment (- -□- -) in a sheep that survived.


3N21
H.I. Antibody
1280
320
80
20
0
Virus titre Log10 p.f.u. per ml.
7
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
Days post-inoculation
Neutralizing Antibody
1·0
0·5
0

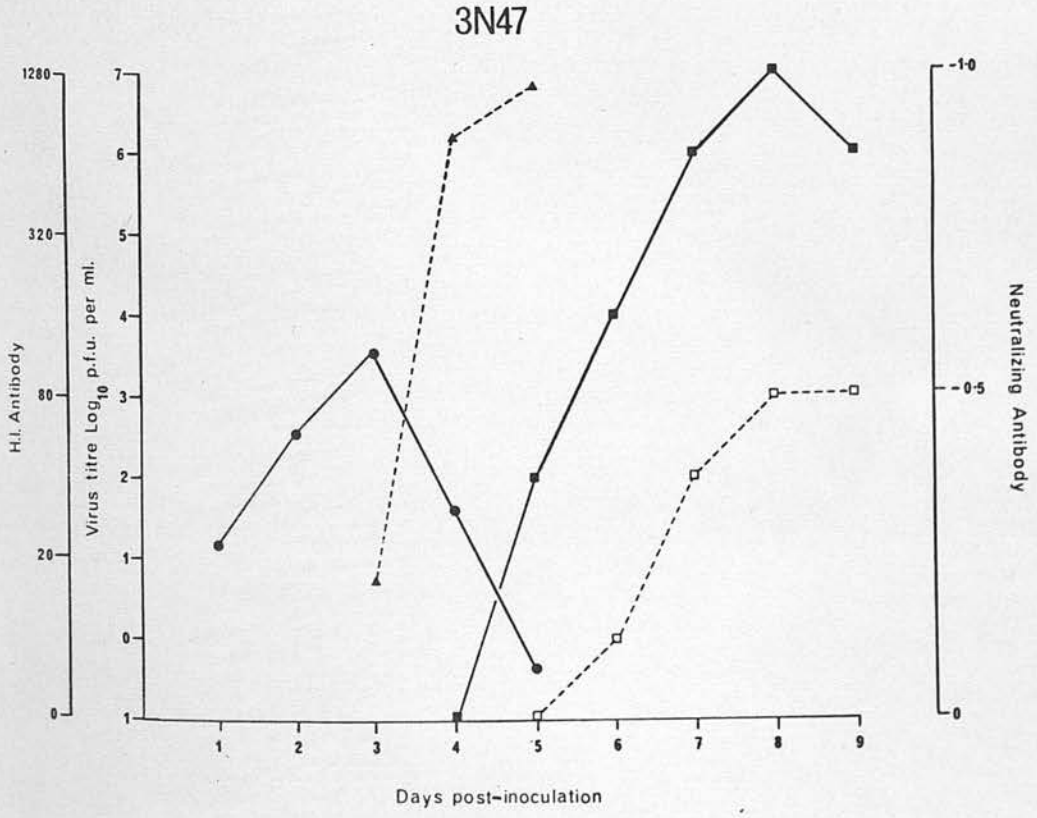
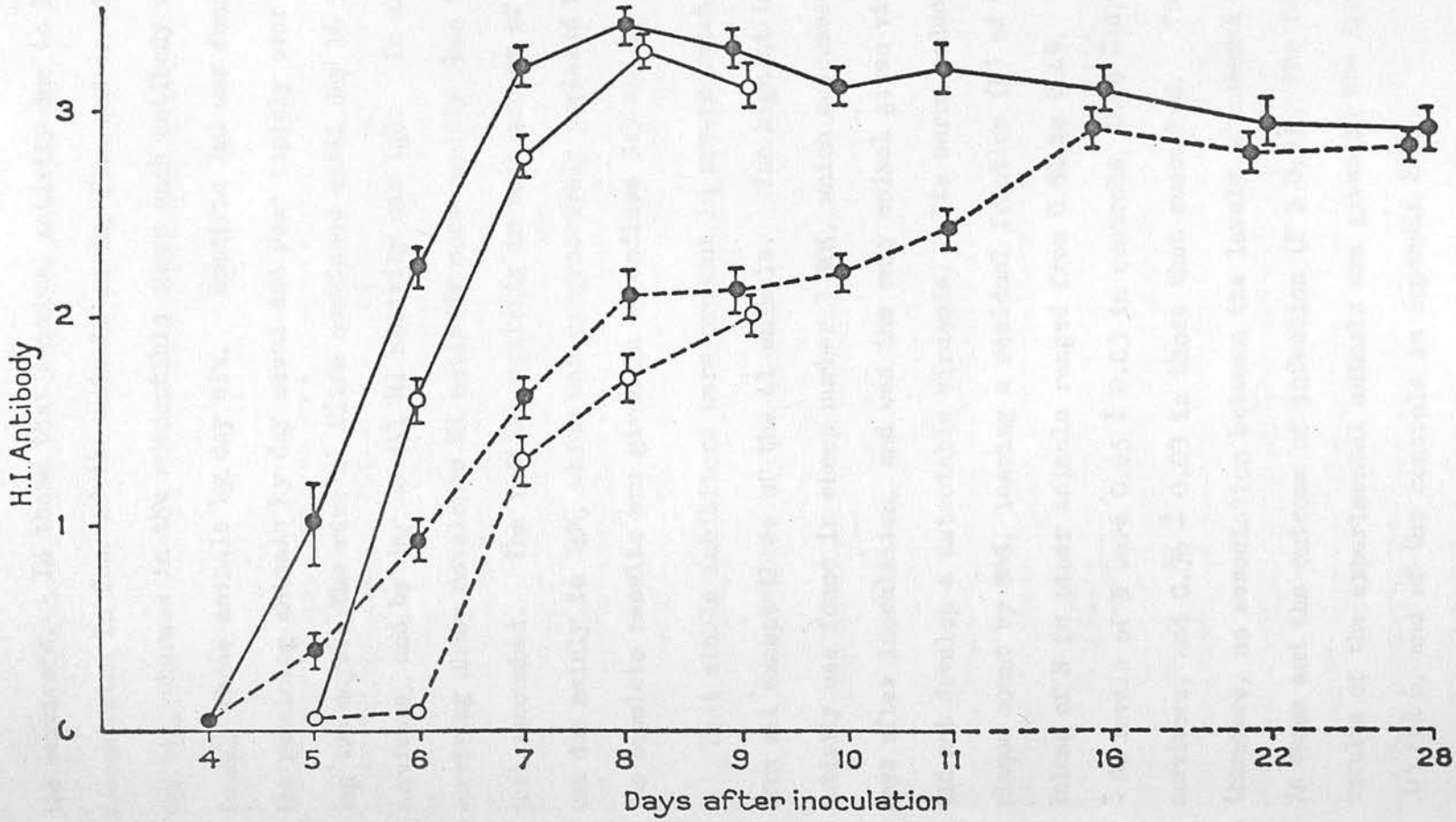

3N47
H.I. Antibody
1280
320
80
20
0
Virus titre Log10 p.f.u. per ml.
7
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
Days post-inoculation
Neutralizing Antibody
1·0
0·5
0

Fig. 3:4. The development of serum HI antibody in susceptible (o) and surviving sheep (●). Values expressed as mean (± SE) of $\log_{10}$ reciprocal titres in whole serum (——) and following treatment with 2-ME (-----).


H.I. Antibody
0
1
2
3
4
5
6
7
8
9
10
11
16
22
28
Days after inoculation

the survivors. In those that survived activity due to IgG was demonstrated in four of the animals on day five and in nine on day six, whereas in the susceptible sheep such antibody was found in only three animals by day six. Specific IgG was found in all the remaining animals by day seven and rose rapidly over the following two days. The rise in titre continued until day 16 in the survivors, and by day 28 all HI activity was IgG. In short, surviving sheep developed HI antibody more rapidly than those that succumbed. The initial activity in both groups of animals was due mainly to IgM, which was progressively replaced by IgG. The complete results are given in Appendices 3:3 and 4.

Heat stable inhibitors were present in pre-inoculation sera from all except three of the 41 animals. The highest level of activity was found in sheep number 3 NO4, which succumbed seven days after inoculation, and was the only animal given virus which did not develop a detectable viraemia. Its serum reduced the plaque count by 86%, leaving a residual fraction (N) of 0.14. Values of N in other animals ranged from 0.91 to 0.49. The mean $\pm$ SE levels of N were 0.65 $\pm$ 0.03 in controls, 0.69 $\pm$ 0.04 in survivors, and 0.76 $\pm$ 0.03 in those that succumbed. There was, therefore, no association between the levels of natural inhibitors in serum and the outcome of infection ($P > 0.2$). The full results of the experimental animals are given in the Appendices 3:5 and 6, and of the controls in Appendix 2:2.

The high level of natural inhibitors present in the serum of

sheep 3 N04 rendered it impracticable to detect the specific neutralizing activity in the serum from this animal. The results from testing this serum have, therefore, been omitted from the analysis.

Prior to day three neutralizing activity was not detected in any of the sera (Table 3:7; Appendices 3:5, 6). On day four, sera from ten of the 11 that survived specifically neutralized 50% or more of the virus inoculum whereas such activity was only detected in eight of the 18 susceptible animals. The mean values were significantly higher in the survivors ($P < 0.005$) which was also true on day five when sera from all animals showed high levels of neutralizing activity ($P < 0.005$) (Table 3:7). Thus, it is apparent that as with HI antibody, neutralizing antibody developed more quickly in the animals that survived.

Serum was collected periodically from three animals that survived for seven months after challenge and again $2\frac{1}{2}$ years later. Following the initial peaks between the second and third weeks post-inoculation there was a gradual decline in HI antibody over the subsequent four to five months (Fig. 3:5). Thereafter there was no appreciable decline over the subsequent $2\frac{1}{2}$ years.

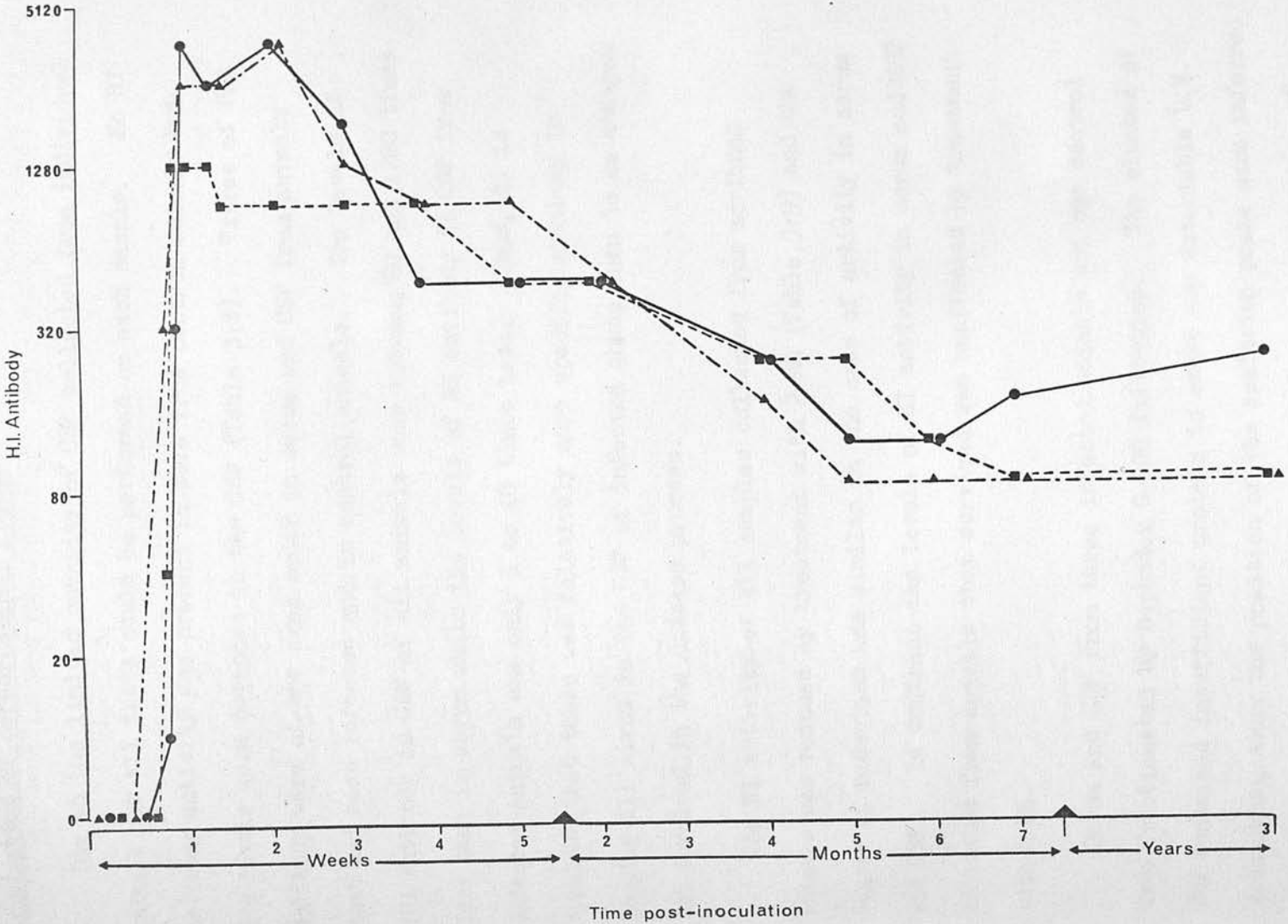
That HI antibody is maintained, probably for life, was further confirmed by bleeding six sheep that had been infected three to five years previously. All had antibody, the titres ranging from 80-640 (Appendix 3:7).

Table 3:7. Development of neutralizing activity in serum, expressed as the mean ± SE S/N values.

Days post inoculation	3	4	5
Control Sheep	-0.00 ± 0.03	+0.10 ± 0.12	+0.08 ± 0.05
Surviving Sheep	-0.12 ± 0.05	-0.73 ± 0.04	-0.94 ± 0.01
Susceptible Sheep	-0.08 ± 0.02	-0.42 ± 0.06	-0.90 ± 0.01
Difference[+]	$P < 0.4$	$P < 0.005$	$P < 0.01$

[+] Difference between survivors and susceptibles as calculated by the Student t-test.

Fig. 3:5. The duration of the serum HI antibody response to louping-ill virus in three sheep following s.c. inoculation.


5120
1280
320
80
20
0
H.I. Antibody
1
2
3
4
5
2
3
4
5
6
7
3
Weeks
Months
Years
Time post-inoculation

Examination of cerebrospinal fluid:

Due to the limited quantity of CSF collected from individual animals not all tests could be performed on each sample. No HI antibody activity was present in sera from control animals but low levels were detected in the CSF (Table 3:8). Titres of PHA antibody were of the same order in serum and CSF from animals that had been infected and in control animals. The levels of PHA antibody in CSF of all animals were between 80 and 1280 times less than in serum while the levels of HI antibody in CSF from infected animals was only 5 to 40 times less. Thus, it is apparent that there was relatively more specific antibody to louping-ill virus in the CSF of infected sheep than to an antigen not involved in the disease process.

The HI activity of all samples collected from moribund animals was reduced by treatment with 2-ME (Table 3:9) and the degree of reduction was similar in the case of activity in serum and CSF. In contrast the levels of HI activity in serum and CSF collected from animals that survived was unaffected by treatment with 2-ME.

Serum and CSF from three infected animals and one control were fractionated by Sephadex G-200 filtration. The eluates of CSF contained insufficient protein to cause any detectable U.V. absorption, thus the position of the exclusion peaks were inferred relative to the position of the peaks following filtration of serum. No HI activity was detected in the eluates from the control

Table 3:8. The Reciprocal HI antibody titre to louping-ill virus and PHA antibody to egg albumen in serum and CSF.

Animal	Days after inoculation	HI Serum	HI CSF	HI Ratio	PHA Serum	PHA CSF	PHA Ratio
3N22	7	>1280	>64	20	2560	<2	1280
3N24	7	640	128	5	320	4	80
3N32	8	5120	512	10	320	4	80
3N34	9	>1280	>128	10	5120	32	160
3N11	9	1280	128	10	2560	8	320
3N31	12	1280	32	40	640	2	320
3N20	30	1280	>64	<20	1280	2	640
3N33	77	>320	16	20	160	<2	160
3N37	78	640	32	20	80	<2	80
3N15	Control	<10	2		320	2	160
3N19	"	<10	<2		160	<2	160
3N40	"	<10	2		320	4	80

Table 3:9. The reciprocal HI antibody titre to louping-ill virus in whole serum and C S F and following treatment with 2-Mercaptoethanol.

Animal	Days after inoculation	Serum PBS[/]	Serum 2ME	Serum Ratio	CSF PBS[/]	CSF 2ME	CSF Ratio
3N22	7	> 1280	20	64	48	< 3	> 16
3N08	8	5120	>160	32	192	3	64
3N32	8	5120	> 80	64	384	6	64
3N11	9	> 2560	> 80	32	384	24	16
3N21	9	1280	160	8	192	3	64
3N34	9	> 640	10	64	96	6	16
3N45	9	> 640	> 80	8	96	6	16
3N06	10	>2560	>320	8	192	6	32
3N31	12	1280	320	4	48	12	4
3N02	28	> 1280	>1280	1	96	96	1
3N20	30	1280	1280	1	48	48	1
3N44	30	> 1280	>1280	1	192	192	1
3N18	35	>320	640	< 1	48	48	1
3N47	35	>320	>320	1	192	192	1
3N33	77	>320	>320	1	48	48	1

[/] Samples were treated with PBS as a control.

serum or CSF.

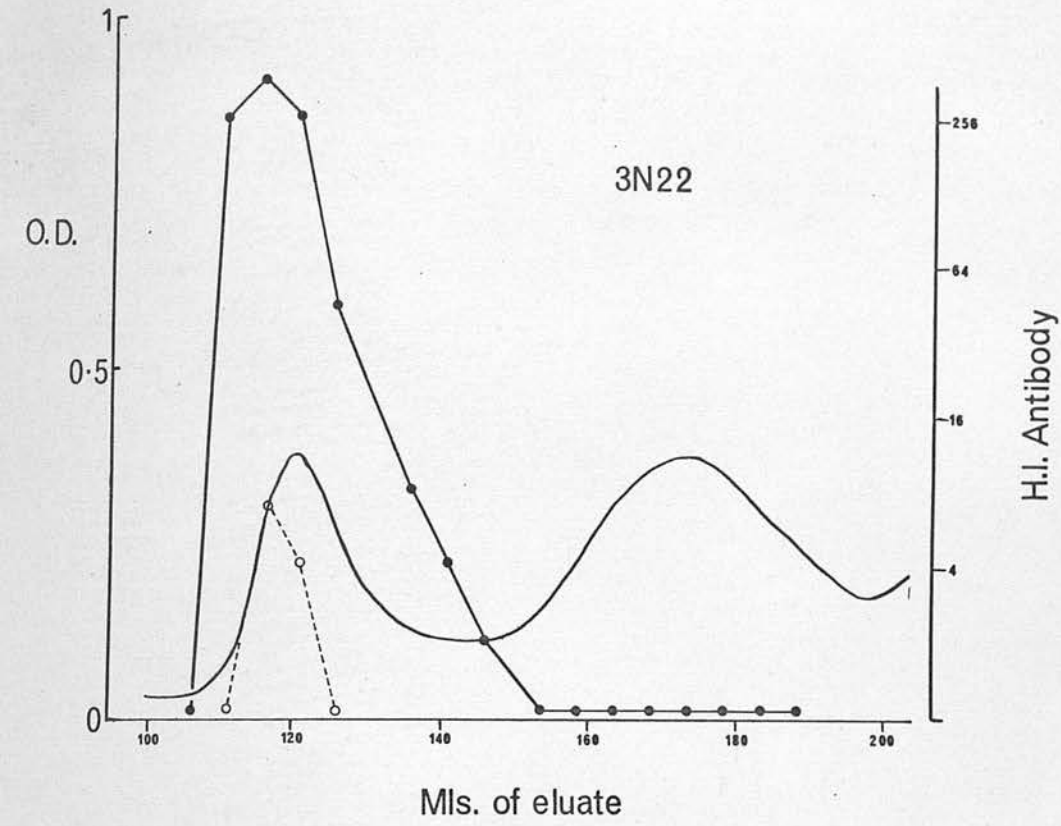
In serum and CSF collected from an animal that succumbed on the 7th day the HI activity was associated with the first exclusion peaks (Fig. 3:6). The activity in the eluates of serum from an animal that was killed on the 12th day was mainly associated with the second peak, and all the activity in the CSF was associated with this peak (Fig. 3:7). In serum and CSF collected from an animal 30 days after infection all activity was associated with the second peaks (Fig. 3:8).

Thus the results of Sephadex G-200 filtration are in agreement with the results of treating serum and CSF with 2-ME. The class of HI antibody present during the acute phase of the disease is largely IgM, whereas that present in surviving animals is largely IgG.

IgM was present in all the CSF samples tested from acute cases but was not detected in samples collected from surviving animals or controls (Table 3:10), CSF from control animals contained some IgG, but the levels were significantly lower than in acute cases ($P < 0.025$) or survivors ($P < 0.01$). The complete results are given in Appendices 3:8 - 11. Total protein levels in CSF from acute cases were also significantly greater ($P < 0.0025$) than those of controls but the difference between controls and survivors was not significant ($P > 0.10$).

Fig. 3:6. The distribution of HI antibody following Sephadex G-200 filtration of serum (—●—) and CSF (- -o- -) collected from a sheep seven days post-inoculation.

Fig. 3:7. The distribution of HI activity following Sephadex G-200 filtration of serum (—●—) and CSF (- - -o- -) collected from a sheep 12 days post-inoculation.


3N22
O.D.
1
0·5
0
100
120
140
160
180
200
256
64
16
4
H.I. Antibody
Mls. of eluate

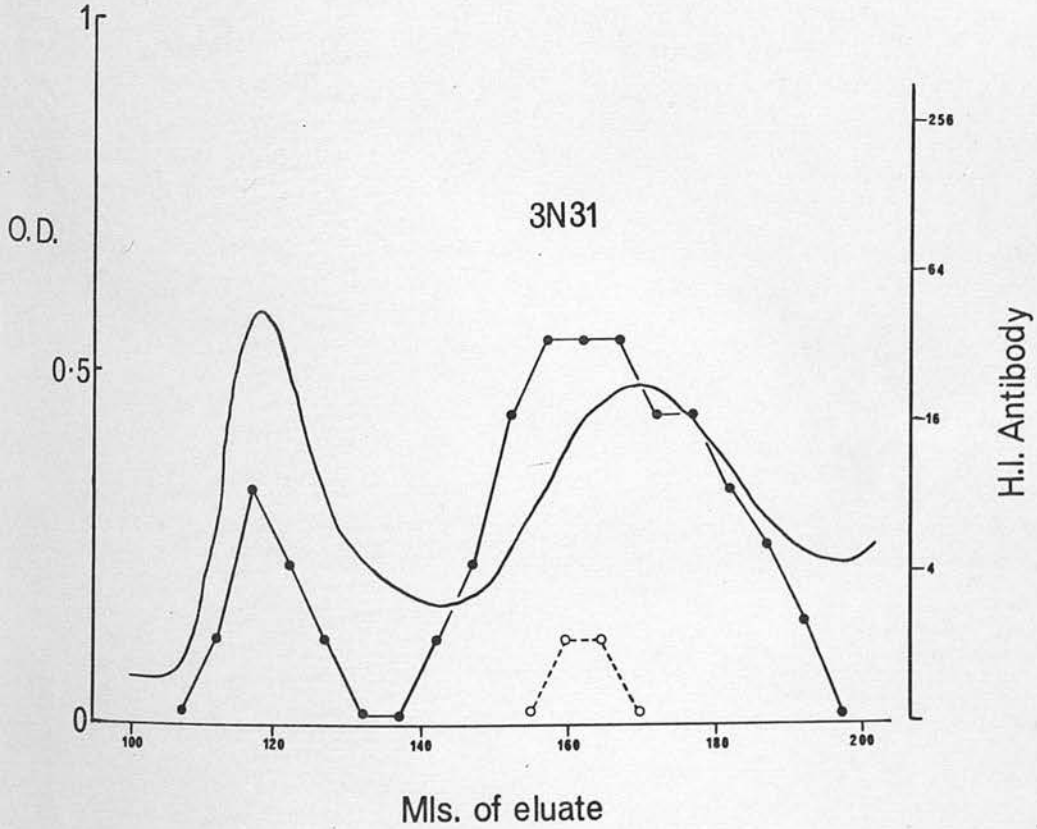

3N31
O.D.
1
0·5
0
100
120
140
160
180
200
256
64
16
4
H.I. Antibody
Mls. of eluate

Fig. 3:8. The distribution of HI antibody following Sephadex G-200 filtration of serum (—●—) and CSF (- - -o- -) collected from a sheep 30 days post-inoculation.

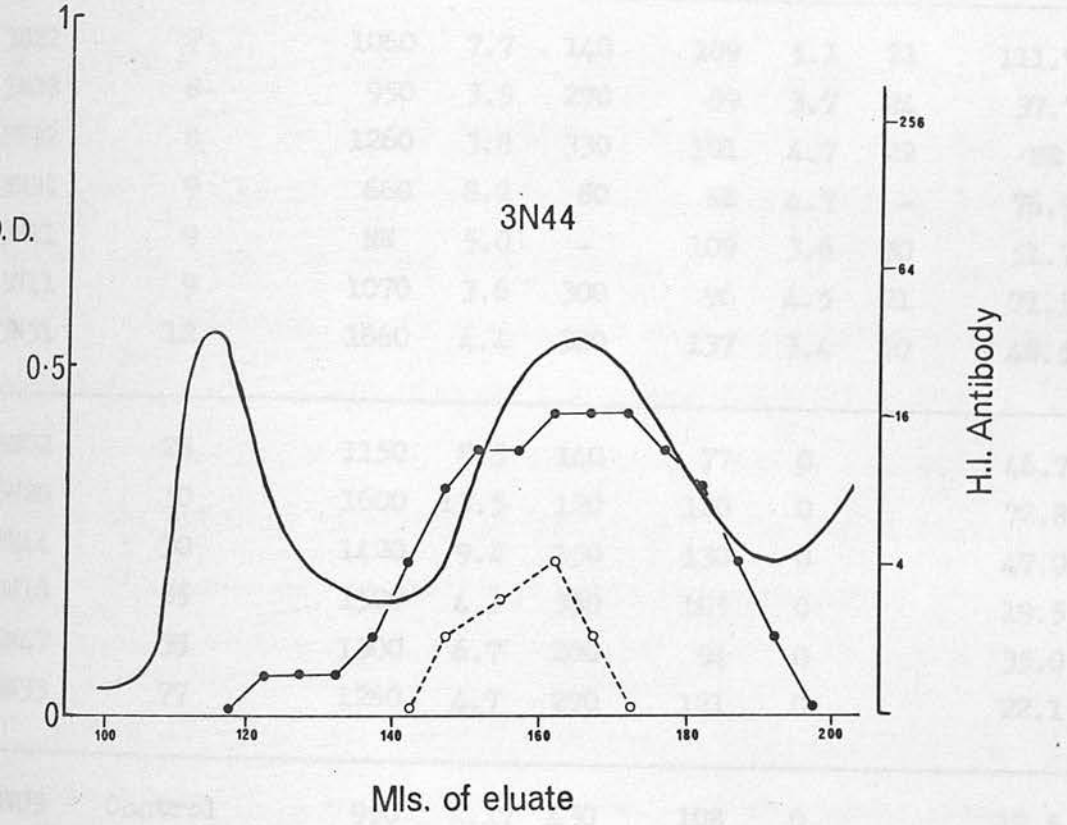

1
0·5
0
O.D.
3N44
256
64
16
4
H.I. Antibody
100
120
140
160
180
200
Mls. of eluate

Table 3:10. Total levels of immunoglobulin detected in sheep serum and CSF and protein in CSF.
Expressed as mg per 100 ml.

Animal	Days after inoculation	IgG			IgM			Protein in CSF
		Serum	CSF	Ratio	Serum	CSF	Ratio	
3N22	7	1060	7.7	140	109	5.1	21	111.9
3N08	8	950	3.5	270	89	3.7	24	37.7
3N32	8	1260	3.8	330	101	4.7	22	NE
3N34	9	660	8.2	80	NE	4.7	-	76.9
3N21	9	NE	5.0	-	109	3.6	30	51.7
3N11	9	1070	3.6	300	96	4.5	21	71.3
3N31	12	1660	4.4	380	137	3.4	40	48.5
3N02	28	1150	8.3	140	77	0		46.7
3N20	30	1600	13.5	120	120	0		72.8
3N44	30	1420	9.2	150	130	0		47.0
3N18	35	1500	4.3	350	105	0		19.5
3N47	35	1300	6.7	200	94	0		35.0
3N33	77	1260	4.7	270	121	0		22.1
3N03	Control	950	2.17	430	108	0		12.5
3N23	"	950	3.8	250	105	0		38.1
3N42	"	2160	3.6	600	128	0		19.0
3N43	"	980	3.5	260	NE	0		22.9
3N46	"	1020	2.8	370	153	0		17.4

NE = Not examined 0 = No immunoglobulin detected.

DISCUSSION AND CONCLUSIONS

The results of these experiments are discussed in detail in Chapters 6 and 7. Only the main points are therefore considered here.

In the first experiment the SB/526 isolate appeared to be the more virulent of the two isolates. Neurological symptoms developed in three of four sheep injected s.c. with SB/526 whereas only slight symptoms were observed in one of four given SB/527. In addition, sheep developed a much more marked viraemia following i.c. inoculation with SB/526 than with SB/527. The degree of viraemia following i.c. inoculation may in some way reflect the inherent virulence of the virus. The SB/526 isolate was therefore selected as challenge virus for subsequent experiments.

Severe signs of neurological dysfunction developed in all sheep inoculated i.c. with either isolate. These animals became moribund prior to the development of detectable serum HI antibody which suggests that antibody plays no part in the disease following this route of inoculation.

Following s.c. inoculation in the second experiment the mean and maximum levels of viraemia detected in the animals that survived tended to be lower than in those that developed severe disease. The viraemia also persisted longer in the animals that succumbed. The decline of the viraemia was associated with the

appearance of both HI and neutralizing antibody, both of which appeared earlier in the survivors. There was, therefore, a definite association between the early appearance of antibody and survival.

The initial HI response in all animals was due to IgM antibody whereas IgG class antibody appeared later. The response of sheep following inoculation with louping-ill virus is therefore the same as following injection with other antigens (Merriman and Rice, 1969; Jonas, 1969).

Pre-existing natural inhibitors were present in the serum of all but three animals and did not obviously affect the outcome of infection. The highest level of activity was detected in a susceptible animal; subsequent studies (Chapter 4) suggested that this animal may have had residual colostrum derived antibody. Natural inhibitors have been found in sera to a variety of viruses and may exhibit a high degree of specificity (McFerran, 1962; Pagano, Gilden and Sedwick, 1965; Zilka, Kawaklova, Vicari and Archetti, 1968) but this does not however imply prior exposure to viral antigen (Koprowski, 1946). Ginsberg (1960) in reviewing their role concluded that in general they were of little significance in the phenomenon on non-specific resistance which is in agreement with the present results.

Virus was present in the brains of all sheep that succumbed but was not demonstrated in the survivors. The highest titres of virus were associated with the brain stem, the area in which

the most severe histological lesions occur (Brownlee and Wilson, 1932).

HI antibody was invariably present in the CSF of animals that had been infected, which was associated with IgM in the susceptible animals and with IgG in the survivors that were killed after 28 days. This was reflected in the total quantity of immunoglobulins present in CSF, IgM only being detected in the animals that succumbed. The relative proportion of HI antibody present in CSF was greater than to an antigen not involved in the disease process. The data all indicate that antibody specific to louping-ill virus was produced locally in the CNS.

In conclusion, the temporal association between the initiation of infection and the appearance of serum antibody was critical to the outcome. It is probable that the humoral response was reflected by local production and release of antibody in the CNS, the antibody so released limiting further dissemination of virus within the CNS. The rapidity with which antibody was released into the CSF therefore appeared to determine the extent of cellular damage in the CNS. A rapid response resulted in only limited involvement in the absence of clinical disease, while a slower response resulted in more extensive damage and clinical louping-ill.

CHAPTER 4

EXPERIMENTAL LOUPING-ILL IN LAMBS

INTRODUCTION

The greater susceptibility of immature animals to infection with togaviruses has been shown with a number of host-virus combinations (Lennette and Koprowski, 1944; Libíková, 1966; Cole and Wisseman, 1969b; Cole, Wisseman and Nathanson, 1973).

The reaction following louping-ill virus infection of immature hamsters (Doherty, 1969a) and guinea pigs (Zlotnik et al, 1971) has also been found more pronounced than in adult animals. In addition, it is generally considered that young lambs are more susceptible than adult sheep, though concrete evidence is lacking (Brownlee and Wilson, 1932; Smith, 1969). In louping-ill endemic areas many lambs are born to immune dams and colostral antibody is transferred (Williams and Thorburn, 1961; Brotherston, Bannatyne, Mathieson and Nicolson, 1971). It is therefore probable that under natural conditions a proportion of lambs will be challenged while colostral antibody is still present.

It appeared desirable to investigate these aspects of infection. In the first experiment four-day-old lambs were challenged by both the i.c. and s.c. routes. Experiments designed to investigate the effect of colostral antibody to louping-ill virus

on the course of infection employed lambs five-to nine-weeks and five-to six-months old.

PART I - The response of four-day-old lambs.

MATERIALS AND METHODS

Twelve lambs born on the institute farm were allowed to suckle for the first three days of life and were then removed to pens fitted with infrared-heater lamps and maintained on a milk-substitute diet.[1.] The lambs were inoculated on the fourth day of life. Following inoculation, animals that developed severe signs of neurological dysfunction were killed _in extremis_ and the survivors were killed on day 20 post-inoculation.

Groups of four lambs were each inoculated i.c. (0.5 ml) or s.c. (5.0 ml) with the SB/526 isolate of virus. Two lambs were inoculated i.c. with control material and two were inoculated s.c. The virus inoculum was titrated by i.c. inoculation of mice immediately after inoculating the experimental animals. Lambs that were inoculated i.c. were found to have been given $10^{6.40}$ mouse i.c. ID_{50} and lambs inoculated s.c. received $10^{7.40}$ mouse i.c. ID_{50}.

Antibody and viraemia were detected using the techniques employed in the first experiment with susceptible sheep (Chapter 3 Part 1). In addition the distribution of virus in blood collected terminally from two lambs that had been inoculated i.c. was

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1. Nutrilamb, Scottish Agricultural Industries, Ltd., Edinburgh.

investigated. Blood was collected in the usual way but in addition 10 ml was withdrawn into an evacuated container to which 40 i.u. of heparin in Hank's BSS had been added. Plasma was separated by centrifugation at 2,000g at +4° C for 20 minutes. The plasma was removed and the cells were washed three times with chilled Hank's BSS. Two aliquots of each of the plasmas and cells were stored at -70° C until screened and titrated in mice.

RESULTS

All lambs inoculated i.c. developed severe neurological clinical signs which were identical to those observed in similarly inoculated sheep, and were killed between 92 and 116 hours after inoculation (Table 4:1). This was a significantly shorter interval than when sheep were challenged by the same route ($P < 0.02$).

Two of the four lambs inoculated s.c. reacted clinically; one (106) was killed when moribund 171 hours post-inoculation and another (641) became ataxic from day 10 - 15. Thus two of the four lambs inoculated s.c. reacted clinically. None of the control animals showed signs of ill-health.

Following i.c. inoculation viraemias tended to be more intense than those found in sheep inoculated by the same route; all lambs circulated virus at levels greater than 10^3 mouse ID_{50} per ml and were still viraemic at death (Table 4:1). As was

Table 4:1. The viraemia* in four-day-old lambs following intracerebral and subcutaneous inoculation with louping-ill virus.

Hour post-inoculation	Lamb no. i.c. 109	110	642	107	s.c. 106/	641	108	104
6	N	N	N	N	N	N	N	N
18	N	N	N	N	T	T	T	N
30	N	N	N	T	T	T	N	T
42	T	T	T	T	N	1.7	T	T
54	1.4	T	T	2.6	2.5	2.3	T	T
66	1.2	T	1.4	3.8	2.7	0.8	T	2.6
78	2.8	2.8	2.4	5.2	3.3	T	1.5	1.5
90	2.8	3.0	2.2	4.6	2.6	T	2.3	1.7
102	(92)	(94)	(97)	3.7	2.2	N	1.5	T
114				2.2	T	T	2.4	T
126				(116)	T	N	1.2	N
138					T	N	2.5	N
150					T	N	T	N
162					N	N	T	N
174					N	N	N	N

*Expressed as $\log_{10}LD_{50}$/0.03 ml of original blood.

T = Trace N = no virus detected ( ) = Time at which moribund animals were killed.

/ Sheep 106 was killed in extremis 217 hours after inoculation. The other animals survived.

observed in sheep the peak level of viraemia recorded following s.c. inoculation was greatest in the animal that succumbed. Most of the infectivity in the blood collected from two of the lambs that were killed following i.c. inoculation was associated with the plasma while only small amounts were found in the washed cells (Table 4:2).

HI antibody was not detected in sera collected from any of the s.c. inoculated animals on day five but was present by day ten (Table 4:3). Both heat and 2-ME treatments indicated that most of the activity in the ten and 15 day sera was due to IgM which was still present on day 20 in the three surviving lambs. No antibody was detected in the sera from the lambs inoculated i.c. or with control material .

PART 2 - The effect of colostral antibody.

MATERIALS AND METHODS

Lambs were inoculated s.c. with the SB/526 isolate and the methods of detecting viraemia and antibody were the same as those employed in the second sheep experiment (Chapter 3, Part 2). The lambs were the progeny of ewes that had been immunised with an inactivated cell culture vaccine (Brotherston and Boyce, 1969, 1970) and were selected from groups which were being studied in relation to the decline of colostral antibody (Brotherston, personal communication).

Table 4:2. The distribution of the virus in the blood of lambs following inoculation with louping-ill virus.

Lamb No.	Whole blood	Plasma	Cells
109	2.8*	3.5	Trace
110	3.0	4.0	0.6

*$\log_{10}ID_{50}$/0.03 ml.

Table 4:3. Reciprocal HI antibody titres to louping-ill virus in four-day-old lambs following subcutaneous inoculation; activity was tested in whole serum and following treatment with heat and 2-ME.

Treatment	Day post inoculation	Lamb no. 106	641	108	104
+PBS	5	<10	<10	<10	<10
	10	640	320	640	>640
	15	(9)	640	>2560	2560
	20		320	320	2560
Heat	5	<10	<10	<10	<10
	10	>80	>10	20	0
	15		80	10	>40
	20		80	40	>320
2-ME	5	<10	<10	<10	<10
	10	80	10	20	10
	15		40	>160	>160
	20		>80	80	640

Titres expressed as reciprocals.

( ) = Day of terminal sample from moribund animal.

+ treated with PBS as a control.

Pilot experiment:

To establish the level of colostral antibody that would modify infection, a pilot experiment was performed using 12 lambs aged five to nine weeks, that possessed a range of antibody titres. The reaction to challenge was assessed on the basis of the HI antibody response only. The age of the lambs and the titres of colostral HI antibody present when challenged are presented in Table 4:4. Four of the lambs had titres of 1/160, four had titres of between 1/10 and 1/40 and four had no antibody when challenged, but these lambs had had titres of 1/20 within ten days of birth (Brotherston, personal communication). Sera from the last four lambs were also tested for neutralizing activity and the N values were found to be 0.0026, 0.0016, 0.1242 and 0.1904 in lambs IV63, 73, 76 and 77 respectively.

All the lambs were inoculated s.c. with $10^{7.08}$ p.f.u. of the SB/526 isolate. They were bled for serum on the 6th, 10th, 13th and 24th days and the HI activity determined in untreated specimens and following 2-ME treatment.

Definitive Trial:

Twenty five lambs were selected on the basis of their HI antibody status and consisted of the following five groups of five lambs each:-

Group 1 lambs had HI antibody at a titre of 1/20.

Group 2 lambs had been first found negative 20 days before

Table 4:4. The reciprocal HI antibody titres in lambs that received colostral antibody and were challenged with louping-ill virus. Sera were tested without treatment and following treatment with 2-ME.

Lamb No.	Age in weeks	Day post-inoculation									
		-2		+6		+10		+13		+24	
		+PBS	2-ME	PBS	2-ME	PBS	2-ME	PBS	2-ME	PBS	2-ME
IV92	5	640	>640	640	640	>320	>320	>320	>320	320	320
IV57	8	320	>320	320	320	160	160	160	160	80	80
IV52	9	160	160	80	80	80	80	80	80	40	40
IV56	8	>160	>160	160	160	>80	>80	>80	>80	>40	>40
IV84	7	40	>40	>20	>20	>40	>40	40	40	20	>20
IV67	9	>20	>20	20	80	>80	>20	80	80	>40	>40
IV81	7	20	20	40	>20	>40	>40	40	40	>40	40
IV88	7	>10	>10	>20	<10	40	>20	>20	>20	>20	>20
IV63	8	<10	<10	20	<10	>40	40	20	>20	20	20
IV73	8	<10	<10	10	<10	>40	>10	>10	10	>20	>20
IV76	8	<10	<10	40	<10	>40	>40	160	160	160	160
IV77	8	<10	<10	10	>10	>80	>20	>80	>80	>80	>80

+Treated with PBS as a control.

challenge.

Group 3 lambs had been first found negative 48 days before challenge.

Group 4 lambs had been first found negative 76 days before challenge.

Group 5 lambs had not received colostral antibody to louping-ill virus.

Plasmas were collected for virus assay on the day of inoculation and for the first nine days. Two aliquots of 2.5 ml of each plasma were stored at - 70° C and no attempt was made to test fresh plasma. Blood for serum separation was collected on the day of challenge and on alternate days for the first nine days and on days 14, 21 and 42. Additional serum samples were collected from groups 1 and 5 on days 71, 139 and 186. Whole serum only was tested for HI antibody.

The five lambs in group 1 were again inoculated with virus, 187 days after the initial challenge and the course of infection was followed in the same manner except that serum was collected daily and the HI activity was determined in whole serum and following treatment with 2-ME.

For both the initial challenge and for rechallenge the SB/526 isolate was inoculated s.c. and it was found that on each occasion each lamb received $10^{7.57}$ p.f.u.

RESULTS

Pilot experiment:

None of the lambs involved in the pilot experiment developed signs of ill health. The levels of antibody in the lambs that had titres of 1/160 when challenged declined throughout the period of the experiment (Table 4:4). The antibody titres in serum from the lamb with an initial titre of 1/40 also declined but did rise between days six and ten. The lambs with titres of between 1/10 and 1/20 all experienced an antibody response and 2-ME reduced this activity in the serum of all three lambs on at least one occasion. The lambs that were negative when challenged all experienced a serological reaction which was similar in nature to that observed previously in susceptible animals. The titres did, however, appear to be depressed, which was most marked in the specimens from IV63 and IV73, the pre-inoculation sera of which had the greatest neutralizing activity.

From the above results it was apparent that lambs with high titres of colostral antibody did not react when challenged with live virus, whereas lambs with low levels underwent a suppressed reaction. Lambs that were negative serologically when tested by the HI test, but had high levels of neutralizing activity, subsequently experienced a normal HI reaction which was only slightly suppressed. The degree of suppression being directly related to the level of neutralizing activity in the pre-inoculation

serum.

In the subsequent experiment, therefore, lambs which either had low levels of HI antibody or had become negative after varying intervals of time were selected for challenge.

Definitive trial:

Clinical signs were observed in two of the 25 lambs inoculated with virus in the definitive trial. One animal in group 3 (C/158) became ataxic on day 10 and died on day 11, and in group 4 one animal (C/213) died on day nine having developed clinical signs on day eight. None of the other lambs showed obvious signs of ill health.

No virus was detected in the plasmas of animals in group 1, and only low levels of virus were detected in two of the five lambs in group 2 while the animals in the other three groups all developed viraemias (Table 4:5; Fig. 4:1). Four of the lambs in group 3 tended to have lower levels of virus present in their plasma on the first three days than were detected in the other two groups, and in one animal never exceeded one p.f.u. per 0.2 ml. The viraemias detected in group 4 were of the same order as found in the control group. No virus was detected in any of the plasmas collected subsequent to day six.

The development of HI antibody in groups 1, 2 and 5 are summarised in Fig. 4:2; details of the reactions of all animals are in Appendix 4:1. Four of the animals in group 2 developed

Table 4:5. Levels of viraemia detected in lambs that received colostral antibody and were challenged with louping-ill virus.

Group No.	Lamb No.	Day post-inoculation 1	2	3	4	5	6
2	C/147	N	N	N	N	N	N
Found nega-	149	N	N	N	N	N	N
tive for HI	151	N	N	0.18	0.53	0.26	0.62
antibody 20	199	N	N	N	N	N	N
days before	201	N	N	N	0.64	N	1.30
challenge.	Mean (±SE)	N	N	0.18	0.59 (±0.06)	0.26	0.16 (±0.46)
3	C/143	0.30+	2.34	3.48	5.32	2.89	N
Found nega-	157	1.60	0.42	1.51	3.85	2.60	N
tive for HI	158	N	N	1.74	3.81	4.11	1.64
antibody 48	206	1.30	0.01	0.01	N	N	N
days before	210	1.78	1.46	2.78	4.60	2.81	N
challenge.	Mean (±SE)	1.25 (±0.33)	1.06 (±0.53)	1.90 (±0.59)	4.39 (±0.36)	3.10 (±0.34)	1.64
4	C/185	0.51	2.26	3.04	4.53	2.42	N
Found nega-	190	1.34	2.45	4.26	4.93	2.04	N
tive for HI	193	0.30	2.28	3.56	4.74	1.82	N
antibody 76	213	0.78	2.46	4.34	5.30	3.08	N
days before	224	N	0.26	0.76	1.88	N	N
challenge.	Mean (±SE)	0.73 (±0.23)	1.94 (±0.42)	3.19 (±0.65)	4.28 (±0.61)	2.34 (±0.28)	N
5	C/196	0.51	2.49	3.00	4.28	2.83	N
No colostral	197	1.78	2.01	2.86	4.28	2.76	N
HI antibody	198	1.90	1.90	2.15	2.60	1.78	N
received.	226	1.45	2.93	4.04	4.56	N	N
	291	N	N	0.81	2.32	2.11	N
	Mean (±SE)	1.41 (±0.32)	2.33 (±0.24)	2.57 (±0.54)	3.68 (±0.47)	2.37 (±0.25)	N

N = No virus detected.

\+ Log_{10} p.f.u./0.2 ml.

Fig. 4:1. The mean ($\pm$ SE) level of viraemia of lambs that circulated virus following inoculation with louping-ill virus. Groups 2, 3 and 4 received colostral HI antibody to louping-ill virus; 2 (—▵—) first became negative 20 days before challenge, 3 (—▲—) first became negative 48 days before challenge, 4 (-·-■·-·) first became negative 76 days before challenge. Group 5(- -●- -) did not receive colostral antibody to louping-ill virus.

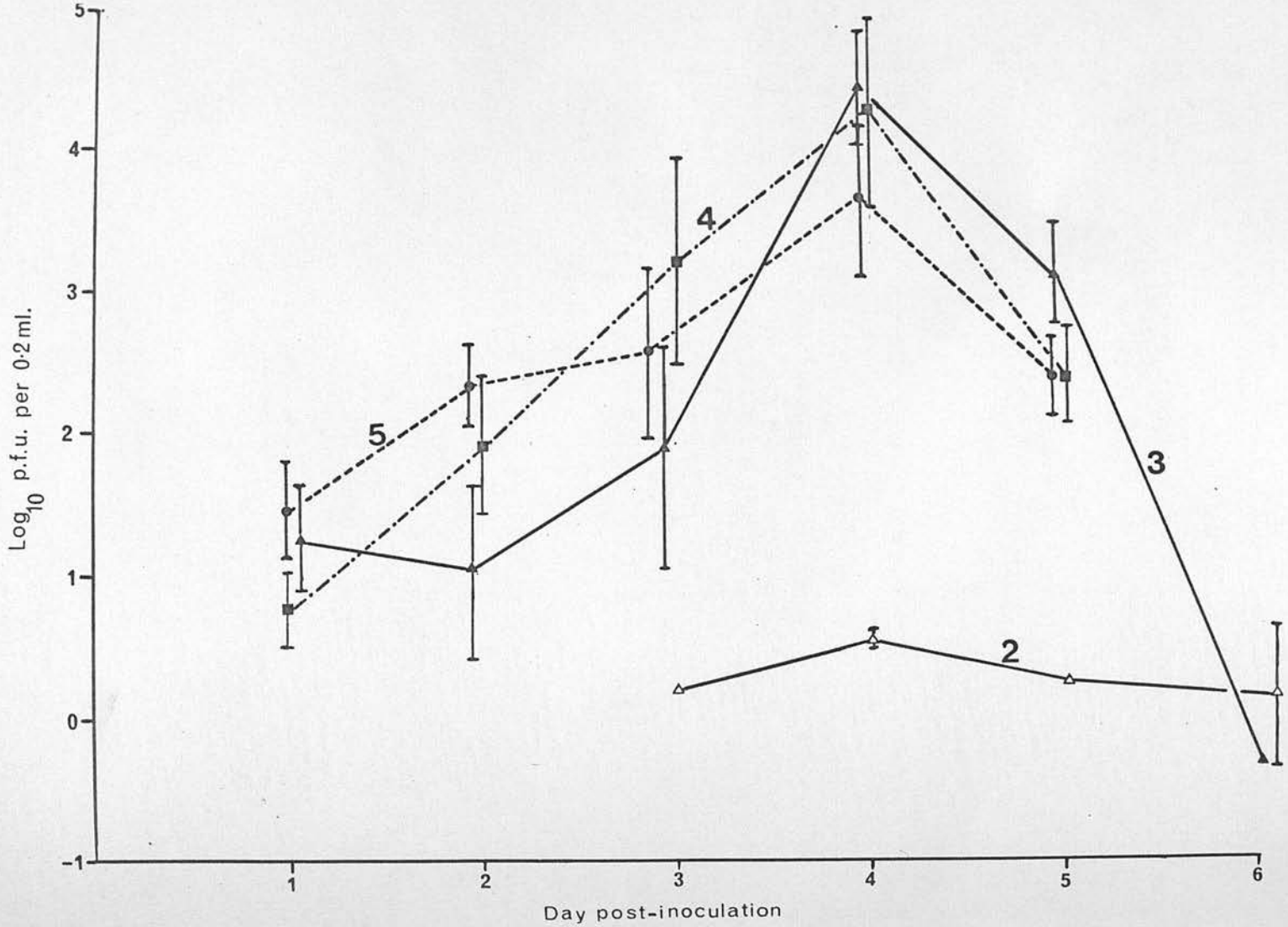
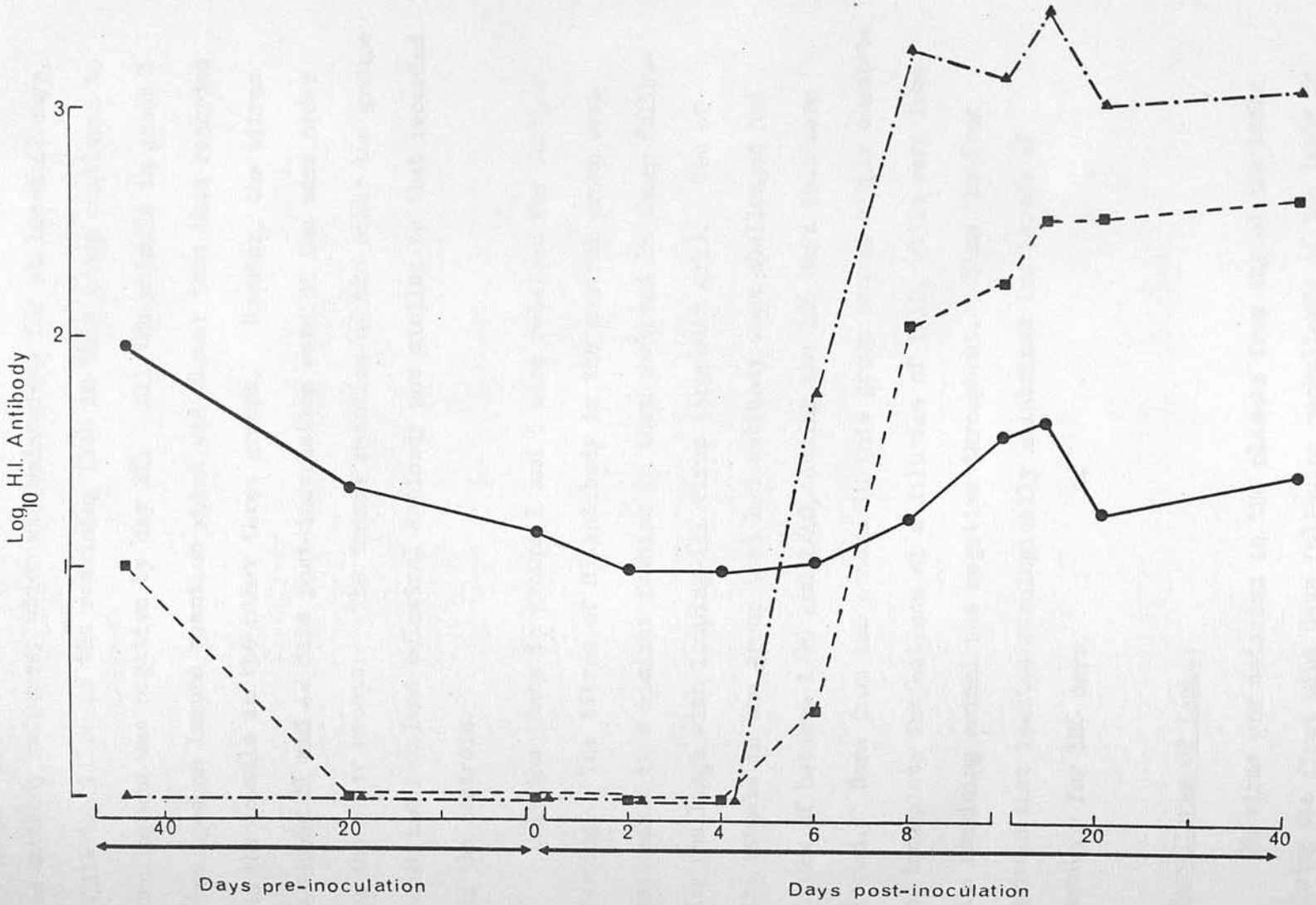

5
4
3
2
1
0
−1
Log10 p.f.u. per 0·2 ml.
1
2
3
4
5
6
Day post-inoculation
5
4
3
2

Fig. 4:2. The mean HI antibody titre in lambs that had colostral antibody when challenged (—●—), that had first become negative 20 days before challenge (- - ■ - -) and in control lambs (-·-·▲-·-·).


3
2
1
Log10 H.I. Antibody
40
20
0
2
4
6
8
20
40
Days pre-inoculation
Days post-inoculation

an antibody response, which was maintained for at least 42 days, while the titre of the remaining lamb in this group continued to decline and was negative by day 21. All the animals in group 2 developed an immune reaction which was slower than that recorded in the animals in the other three groups. However, the titres achieved 21 and 42 days post-inoculation were of the same order as the other groups. The immune reaction of the other two groups which had received colostral antibody was similar to that recorded in the controls.

Only the lambs in groups 1 and 5 were retained for further testing. The titres of HI antibody in the control group were maintained in a similar fashion to that observed in sheep following challenge with louping-ill virus (Appendix 4:1). Two of the animals in the group that had antibody when challenged had titres of between 1/20 and 1/40, during the 186 days that were tested. Sera from two others in this group caused either complete or incomplete inhibitions at a dilution of 1/10, while sera from the remaining animal was negative throughout. Thus the four animals that reacted serologically maintained low levels of antibody for 186 days.

Rechallenge of lambs:

No virus was detected in the plasmas from any of the lambs during the first nine days following rechallenge of the lambs in group 1.

Table 46. The reciprocal HI antibody titres in lambs that were rechallenged 187 days after initial exposure. Sera were tested without treatment and following treatment with 2-ME.

Day post inoculation	Lamb No. C/155		C/156		C/191		C/192		C/195	
	+PBS	2-ME	PBS	2-ME	PBS	2-ME	PBS	2-ME	PBS	2-ME
1	40	40	20	>20	10	10	10	10	<10	<10
2	40	>20	20	20	10	10	10	10	<10	<10
3	40	40	40	40	10	10	10	10	<10	<10
4	80	80	>40	80	>20	20	>20	>20	<10	<10
5	80	80	80	80	>80	160	>80	>80	<10	<10
6	160	>80	160	>160	320	320	320	320	<10	<10
7	320	>160	320	>160	>320	>320	640	640	<10	<10
8	160	>160	320	320	>320	>320	320	640	<10	<10
9	>160	>160	320	320	640	>320	640	>320	Trace	10
14	>320	>320	640	640	640	>320	>1280	>640	10	10
21	>320	>320	640	640	>320	>320	>640	>640	10	10
28	320	320	320	>160	160	160	320	320	10	10
35	320	>320	320	320	160	160	320	>160	10	10

+ Treated with PBS as a control.

Four of the animals experienced an increase in HI antibody titre by day four while the other remained negative (Table 4:6). The titres in these four continued to rise and achieved maximum levels of between 1/320 and 1/1280. The titre of none of these sera was affected by 2-ME. That the 2-ME treatment was effectively reducing IgM was confirmed by testing two sera from clinical cases at the same time. The titres in both untreated sera was 1/1280 and in the treated specimens was 1/160 and 1/40 respectively.

The remaining animal did not develop HI antibody till day 14 when a titre of 1/10 was recorded, which was maintained till day 35 when the experiment was terminated.

DISCUSSION AND CONCLUSIONS

Following i.c. inoculation four-day-old lambs became moribund after a shorter period than did adult animals inoculated by the same route indicating a greater susceptibility of the young lamb to i.c. challenge. This conclusion is also supported by the fact that the viraemias that developed in the young lambs inoculated i.c. tended to be more intense than found in sheep inoculated with the same isolate.

Clinical louping-ill was observed in two of the four lambs challenged s.c. and one of these became moribund which is of the same order as observed in adult animals inoculated by this route.

The nature of the viraemia and antibody response in both four-day-old lambs and adult animals inoculated by this route was similar. From these limited experiments, therefore, there is no evidence to support the contention that very young lambs are more susceptible to s.c. infection with louping-ill virus than adult animals.

Maternal antibody was effectively transferred to lambs and was seen to modify the course of experimental infection. Infection in lambs with high titres of antibody experienced infection in the absence of a viraemia. Evidence of infection in such cases was found in the development of a suppressed antibody response and or sensitization to virus antigen.

The antibody response following rechallenge in four of the five animals that had antibody when initially challenged was more rapid and in the absence of IgM. Such a response is typical of an anamnestic reaction. In the remaining case the immune response was delayed and very much reduced to that observed in any previous case. This animal also failed to produce IgM. Thus, it too, appeared to have been sensitized by the initial exposure. Animals that had recently become negative as measured by the HI test developed viraemias of low intensity and a slightly delayed antibody response which however achieved levels similar to control lambs after 21 days. Lambs that had been negative for longer periods of time developed normal reactions.

In louping-ill endemic areas it is probable that lambs which receive colostral antibody will be completely refractory to infection

during the spring tick rise. It is unlikely that the level of maternal antibody will fall to a level where sensitizing infection could occur before the main period of tick activity has passed. In areas where there is an autumn recrudescence of ticks, lambs possessing a range of maternally derived antibody will be present and it is possible that a proportion may experience sensitizing infection during this period.

CHAPTER 5

EXPERIMENTAL LOUPING-ILL IN RED GROUSE

INTRODUCTION

In addition to domestic species there was evidence that louping-ill virus infection occurred naturally in several feral species: red deer (Cervus elaphus) (Dunn, 1960), wood mouse (Apodemus sylvaticus) and common shrew (Sorex araneus) (Smith, Varma and McMahon, 1964) and red grouse (Lagopus scoticus) (Williams et al, 1963; Watt et al, 1963). Of these species however only the red grouse has been studied experimentally. Williams et al (1963) infected two grouse, both of which died seven and eight days respectively after infection. No lesions could be detected on histopathological examination of the brains of these two birds and the cause of death remained in doubt.

Observations of grouse populations in certain areas of Scotland suggested that louping-ill virus infection was a serious cause of mortality (Phillips, personal communication). There was evidence, therefore, that infection of red grouse did occur under natural conditions and, in addition, there was a suggestion that the outcome of infection might be fatal. A study of the experimental infection in red grouse was therefore undertaken to establish if the viraemia that developed in this species was of a sufficient

intensity to infect ticks and if infection could have a fatal outcome.

MATERIALS AND METHODS

Reared grouse four to seven months of age were kindly supplied by the grouse team of the Institute of Terrestrial Ecology, Banchory. On arrival the birds were placed in wire-bottomed cages in groups of six to eight. Birds were randomized prior to inoculation and control and infected birds were present in each cage. The grouse were supplied in two separate batches, but as the results of inoculation were similar on each occasion they have been pooled.

Virus and control inocula were injected into the right tarsal pad in 0.03 ml aliquots and it was found that in the first experiment infected birds received $10^{4.30}$ p.f.u. and in the second $10^{4.70}$ p.f.u. of the SB/526 isolate. A total of 37 birds were inoculated with virus and 20 with control material.

Following inoculation the birds were inspected at frequent intervals and specimens were collected as soon as possible after death. Specimens were not collected from any bird that was cold when found. Four surviving birds were killed on day 14 after inoculation and the remaining four were killed on the 19th day.

Blood for virus assay was collected by pricking the wing vein and filling a heparinized[1]. capillary tube. Each capillary

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[1]. Harshaw Chemicals Ltd., Northants, England.

contained approximately 0.08 ml of blood, which was expressed into 0.8 ml of growth medium to give an approximate dilution of 1/10. The diluted specimens were stored at - 70° C until tested for virus by the plaque assay using two plates for each dilution.

For antibody determination an additional capillary tube was filled and expressed into 0.8 ml of 0.85% saline containing 0.5% BA. The cells were sedimented at 1200 g for 15 minutes and the supernatant was removed and stored at - 20° C until tested. Whole blood for serum separation was also collected from a few birds immediately after they had died.

All the birds were sampled before inoculation for antibody and virus assays. Specimens for virus assay were collected at daily intervals for 10 days after inoculation from 27 of the infected birds and 18 of the controls. In addition, 15 of these experimental birds and nine of the controls were sampled for antibody determinations. The remaining ten experimental and two controls were not sampled following inoculation.

On the second day after inoculation additional capillaries of blood were collected from seven birds and expressed into 10 ml of medium. The blood was centrifuged at 2500 g for 15 minutes and the supernatant fluid collected. The cells were washed three times with growth medium then resuspended in 2 ml of medium. The supernatant fluid and the washed cells were stored at - 70° C until assayed for virus.

Sera and plasma were tested for HI antibody following

extraction with kaolin as detailed earlier except that the plasma samples were extracted with an equal volume of the kaolin suspension because the plasmas had been diluted 1/10 at the time of collection; the final dilution of the extracted plasmas was 1/20.

Portions of brain of some of the birds that died and of the eight surviving birds which were sacrificed on days 14 and 19 respectively were collected and stored at - 70° C. These were homogenized and tested for virus content as described earlier.

RESULTS

All the birds inoculated with virus developed clinical signs between the 2nd and 8th days after infection. The typical signs were depression, feather fluffing, anorexia, muscular weakness and regurgitation of crop contents on handling. No signs directly attributable to involvement of the CNS were observed. Of the 27 birds that were bled daily 21 died, and eight of the ten that were not sampled daily also died; infection therefore had a fatal outcome in 29 of the 37 birds given virus (Table 5:1). Nine of the 21 birds that died in the group that was sampled daily succumbed either while being handled or immediately afterwards. Although a similar proportion of the birds in both groups died, the procedure of handling birds daily for the collection of samples appeared to precipitate death. The eight infected birds that survived appeared normal by day ten to 12.

Table 5:1. Cumulative mortality of red grouse (Lagopus scoticus) following peripheral inoculation with louping-ill virus.

Treatment	Total No. Birds	Day post-inoculation 2	3	4	5	6	7	8	9	10	11	12	13
Sampled daily	27	0+	2	4	10	13	15	18	19	20	21	21	21
Not sampled	10	0	0	0	0	1	3	4	6	7	7	7	8
Total	37	0	2	4	10	14	18	22	25	27	28	28	29
Percentage		0	5.4	10.8	27.0	37.8	48.6	59.5	67.6	73.0	75.7	75.7	78.4

+ No. of birds dead.

All the control birds remained healthy throughout the experiment except two that developed typical signs on the 9th and 11th days respectively and both died on the 15th day. These birds were caged with infected birds which were sampled daily, and became viraemic on the 2nd and 6th day respectively. Of a total of 39 birds shown to have become infected 31 (79%) died.

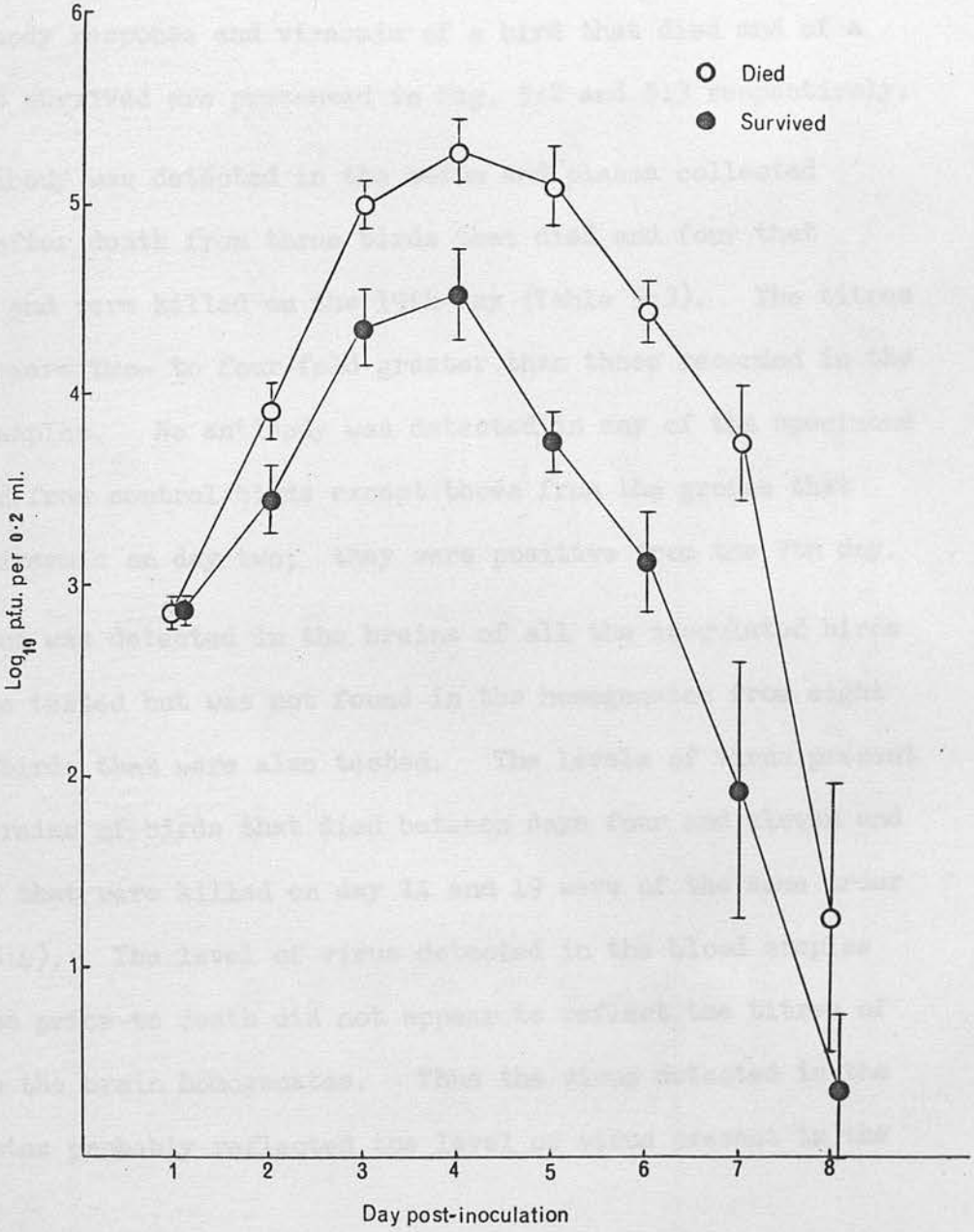
At 24 hours virus was detected in all the infected birds that were tested. Maximum levels of virus were circulated on the 3rd and 5th days and ranged from $10^{4.48}$ to $10^{7.60}$ p.f.u. per 0.2 ml. The mean level of viraemia in birds that died tended to be higher than in those that recovered (Fig. 5:1), and this difference was significant on day three and four ($P < 0.02$), five ($P < 0.005$) and six ($P < 0.001$) (Appendix 5:1). Viraemia was detected on the 7th day in eleven of the twelve remaining birds and in four of eleven and none of eight on the 8th and 9th days respectively. No virus was detected in any of the samples from the control birds except in those from the two that died. One of these was viraemic from day two to eight and the other from day six to ten. This bird had $10^{5.60}$ p.f.u. per 0.2 ml of blood on day ten, at which time sampling was terminated.

The level of virus detected in the washed cells collected from infected birds on the 2nd day of viraemia was 1.5 p.f.u. per 0.2 ml whereas there was $10^{3.26}$ per 0.2 ml in the supernatant fluid.

Six of the birds tested for antibody died before it could be

Fig. 5:1. The mean ($\pm$ SE) level of viraemia in red grouse (*Lagopus scoticus*) that died and in those that survived, following peripheral inoculation with louping-ill virus.

No. of birds tested that: died	21	21	20	18	16	10	7	6
survived	6	6	6	6	6	6	6	6



detected. HI antibody was first detected in the plasmas of four of the eleven birds tested on day five and in all the birds tested on day six (Table 5:2). Maximum levels of antibody were detected between days eight and ten and ranged from 1/5120 to 1/40,960. The antibody response and viraemia of a bird that died and of a bird that survived are presented in Fig. 5:2 and 5:3 respectively.

Antibody was detected in the serum and plasma collected shortly after death from three birds that died and four that survived and were killed on the 19th day (Table 5:3). The titres in serum were two- to four-fold greater than those recorded in the plasma samples. No antibody was detected in any of the specimens collected from control birds except those from the grouse that became viraemic on day two; they were positive from the 7th day.

Virus was detected in the brains of all the inoculated birds that were tested but was not found in the homogenates from eight control birds that were also tested. The levels of virus present in the brains of birds that died between days four and eleven and in those that were killed on day 14 and 19 were of the same order (Table 5:4). The level of virus detected in the blood samples collected prior to death did not appear to reflect the titres of virus in the brain homogenates. Thus the virus detected in the homogenates probably reflected the level of virus present in the brain.

Table 5:2. Reciprocal HI antibody titres in grouse (Lagopus scoticus) plasmas following peripheral inoculation with louping-ill virus.

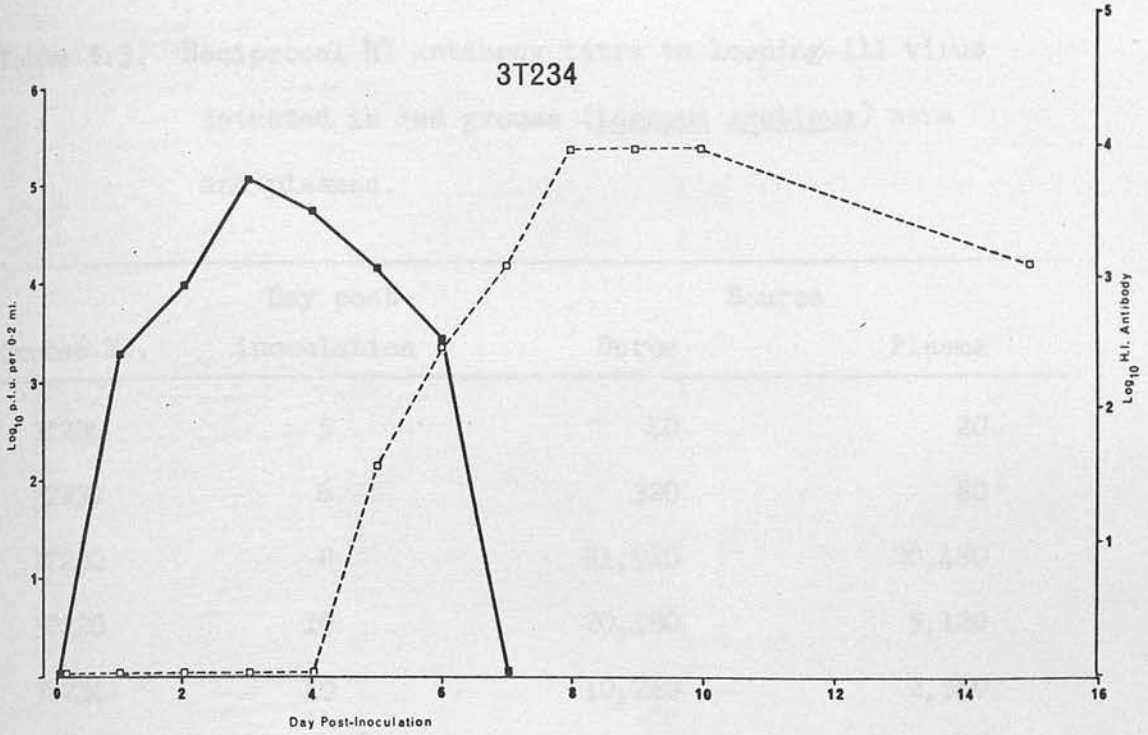
Grouse No.*	Day post-inoculation 4	5	6	7	8	9	10	15
3T126	<20	<20	40	D				
3T206	<20	20	D					
3T226	<20	<20	80	2560	10240	40960	40960	D
3T228	<20	<20	160	1280	40960	40960	40960	5120
3T32	<20	<20	160	1280	20480	D		
3T234	<20	40	320	1280	10120	10240	10240	1280
3T237	<20	<20	80	D				
3T238	<20	20	320	10240	20480	20480	D	
3T243	<20	320	1280	5120	2560	1280	5120	2560

D - bird dead.

* - 6 grouse tested died on or before day 5 and did not develop antibody before death. These birds are not represented in the Table.

Fig. 5:2. The viraemia (—■—) and HI antibody response (- -□- -) in a red grouse (Lagopus scoticus) that survived.

Fig. 5:3. The viraemia (—■—) and HI antibody response (- -□- -) in a red grouse (Lagopus scoticus) that died 9 days after inoculatio


3T234
Log10 p.f.u. per 0·2 ml.
Log10 H.I. Antibody
Day Post-Inoculation
0
2
4
6
8
10
12
14
16
1
2
3
4
5

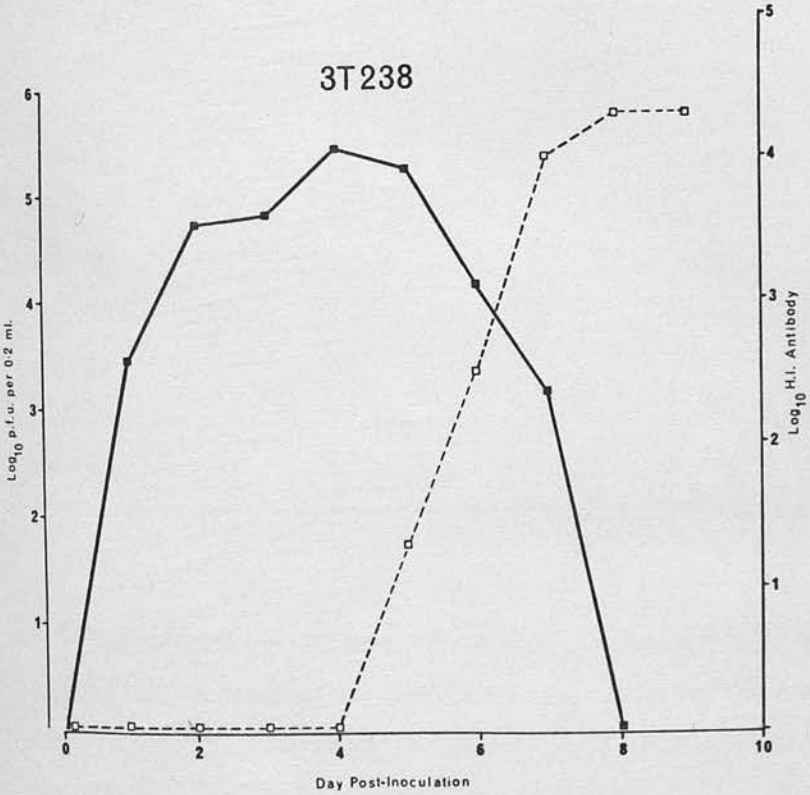

3T238
Log10 p.f.u. per 0·2 ml.
Log10 H.I. Antibody
Day Post-Inoculation
0
2
4
6
8
10
1
2
3
4
5

Table 5:3. Reciprocal HI antibody titre to louping-ill virus detected in red grouse (Lagopus scoticus) sera and plasmas.

Grouse No.	Day post-inoculation	Source	
		Serum	Plasma
3T206	5	40	20
3T237	6	320	80
3T232	8	81,920	20,480
3T228	19	20,480	5,120
3T234	19	10,240	2,560
3T243	19	2.560	1,280
3T246	19	5,120	1,280

Table 5:4. The levels of virus detected in the brains of grouse (Lagopus scoticus) that died or were killed following inoculation with louping-ill virus.

No.	Day post-inoculation	Virus Titre In Brain	Virus Titre In Blood
3T208	4	4.04[+]	5.32[/]
3T236	4	4.06	6.00
3T096	6	4.16	3.75
3T147	6	3.84	4.66
3T237	6	4.34	5.66
3T094	7	3.60	4.72
3T251	7	4.28	NE
3T121	8	4.58	NE
3T140	8	4.38	4.10
3T158	8	4.84	2.75
3T232	8	4.10	0.70
3T255	8	4.45	NE
3T120	9	5.20	NE
3T170	9	4.13	NE
3T238	9	3.75	N
3T259	10	2.62	NE
3T226	11	2.24	N
3T051	14	2.41	N
3T061	14	4.41	N
3T083	14	4.93	N
3T109	14	3.56	NE
3T228	19	2.41	N
3T234	19	2.41	N
3T243	19	3.75	N
3T246	19	5.22	NE

[+] Virus titres expressed as $\log_{10}$ pfu. per 0.2 gm of brain.

[/] Virus titres expressed as $\log_{10}$ pfu. per 0.2 ml of whole blood on the last occasion that samples were collected prior to death.

NE=not examined.

N=no virus detected.

DISCUSSION AND CONCLUSIONS

The mortality rate in 37 birds injected with virus was approximately 78% and there was no evidence that the effect of daily handling for the purpose of bleeding was a contributory factor. All the control birds remained healthy except two that became infected with virus and died. These two birds were caged with infected birds and may have become infected by blood from viraemic birds during the sampling procedure. Louping-ill virus infection must be regarded as a possible natural cause of mortality of red grouse.

The levels of viraemia detected in all birds were in excess of that expected to infect I. ricinus and the levels persisted in most cases for at least six days. Grouse may therefore be regarded as potentially important in the transmission of louping-ill virus to the vector. The viraemias detected in the grouse that survived tended to be lower than in those that died. In addition the decline in the viraemias was associated with the appearance of high titres of HI antibody. Thus as was observed in experimental studies of the infection in sheep there is evidence to suggest that the immune response was essentially protective in nature.

CHAPTER 6

DISCUSSION OF THE PATHOGENESIS OF LOUPING-ILL VIRUS INFECTION

In considering the epidemiology of arthropod-transmitted virus infections there are two main components. Firstly, there is the interaction of the virus with the vertebrate host with respect to the pathological processes of the infection which will be discussed in the present Chapter. Secondly, there is the biological importance of the infection with respect to its potential to transmit to the vector which will be discussed in Chapter 7.

Invasion of the central nervous tissues of sheep following peripheral infection with louping-ill virus has been considered to be of critical importance in determining the outcome of infection (MacLeod and Gordon, 1932; Gordon et al, 1932b; Smith et al, 1964). Factors contributing to the "overwhelming of the blood brain barrier" have been central to the discussion. MacLeod and Gordon (1932) suggested that concurrent tick-borne fever played a major role in facilitating virus to gain access to the CNS. The report by the same workers that 33 of 50 sheep succumbed following peripheral inoculation when tick-borne fever could not have been present (Gordon et al, 1932a) argues against the necessity for such a proposition. Additional factors which might facilitate invasion of the CNS were considered by Smith et al (1964) included age, nutrition, cold and increased blood carbon dioxide.

Nevertheless, Smith and his colleagues maintained that the degree and duration of viraemia determined whether an encephalitic phase developed.

It would now appear certain that virus generally invades the CNS following a s.c. inoculation of susceptible sheep. The regularity with which this occurs suggested that histological examination of the brain following peripheral challenge would be of value in assessing the efficacy of vaccines (Zlotnik, Keppie and Grant, 1970). In histological studies of the brains of sheep that did not succumb, in the experiments described here, lesions were also invariably present (Doherty and Reid, 1971a,b). Further evidence that viral invasion of the CNS is the normal sequence of events, is also provided by the finding of high titres of antibody to louping-ill virus in the CSF of sub-clinical cases. This also appears to be true of the red grouse; virus was detected in the brains of the eight surviving birds sacrificed on days 14 and 19 and marked histological lesions were observed (Buxton and Reid, 1975).

Non-fatal infection of the CNS has been demonstrated in a wide variety of host-virus relationships (Table 6:1); it is therefore evident that many togaviruses regularly gain access to the CNS but may not produce clinical signs. The variable outcome of infection in sheep and grouse cannot be attributed to whether virus invades the CNS or not.

The method of entry into the CNS was not investigated in the

Table 6:1. Experimental systems in which non-fatal togavirus infection of the CNS has been demonstrated.

Vertebrate	Virus	Reference
Mouse	Langat	Seamer and Randles, 1967; Webb, Wight, Wiernik, Platt and Smith, 1968b; Thind and Price, 1969.
	Sindbis	McFarland, Griffin and Johnson, 1972.
	Dengue	Cole, Wisseman and Nathanson, 1973.
	Venezuelan equine encephalomyelitis	Berge, Gleiser, Gochenour, Meisse and Tigertt, 1961.
	Kemerovo	Libíková, 1966. Mayer, 1972.
	Kyasanur forest disease	Price, 1966.
	West Nile	Weiner, Cole and Nathanson, 1970.
Hamster	Kemerovo	Libíková, Erneck and Albrecht, 1965.
	Tick-borne encephalitis	Šimon Slonim and Zavádová, 1966.
	Louping-ill	Doherty, 1969a.
	Venezuelan equine encephalomyelitis	Austin and Scherer, 1971.
Monkey	Japanese B	Nathanson and Cole, 1970a.
	Louping-ill	Ilyenko and Pakrovskoya, 1960.
Rat	Louping-ill	Burnet , 1936.

present studies. No evidence of a preferential site of entry was apparent on histological, or immunofluorescent studies of the brains of the animals that succumbed (Doherty and Reid, 1971a,b). Nor did the distribution of infectious virus in nervous tissue suggest a particular route for viral invasion. Penetration may be concluded to be via the haematogenous route and to occur in a random fashion. In both species studied the viraemia that developed was almost entirely associated with the plasma, thus it is unlikely that virus dissemination was mediated by invasion of infected cell elements (Johnson, 1965). Viral antigens could not however be detected in the vascular-endothelium of sheep that succumbed (Doherty and Reid, 1971b). Studies of other togavirus infections have also failed to demonstrate the presence of virus in endothelial cells; tick-borne encephalitis virus (Albrecht, 1960; 1962; Shi - Gie and Pogodina, 1964), dengue virus (Cole, Wisseman and Nathanson, 1973), Langat virus (Dalton, 1972) Venezuelan equine encephalitis virus (Kundin, 1966) and Japanese B encephalitis virus (Oyanagi Ikuta and Ross, 1969). In only two studies has viral antigen been detected in endothelium; West Nile virus (Kundin, Liu, Hysell and Hamachige, 1962) and Sindbis virus (Johnson, 1965). In explanation of this mainly negative data passive movement of virus across cerebral capillaries has been suggested (Nathanson and Cole, 1970b; Webb, 1970) without virus replication (Albrecht, 1968).

The generally held opinion of invasion of the CNS via the

haematogenous route is that it is determined by the magnitude of the viraemia (Smith et al, 1964; Johnson and Mims 1968). In the present study virus was shown to have gained access to the CNS in every case irrespective of the level of viraemia. In addition following peripheral inoculation, sheep succumbed between the 6th and 12th days after infection while after i.c. inoculation death occurred on days five and six. Maximum viraemia in the s.c. inoculated sheep that succumbed was not found on average until the 4th day, whereas on average severe signs of encephalitis occurred by the 8th day; this interval is shorter than the incubation period following i.c. inoculation. Furthermore, one of the susceptible sheep that succumbed never developed a viraemia at detectable levels and one of the lambs that received colostral antibody and developed a low intensity viraemia also succumbed. In the absence of any evidence that there is a quantitative relationship between the level of viraemia and invasion of the CNS it would appear that virus probably gains access to the brain at an early phase in the infection. With other togaviruses entry into the CNS of mice appeared coincidental with the onset of viraemia e.g. Japanese B encephalitis virus and St. Louis encephalitis virus (Peck and Sabin, 1947; Huang and Wong, 1963). Factors relating to the control of infection within the CNS therefore must dictate the variable outcome of infection.

Two principal mechanisms are generally considered important in recovery from virus infection; interference and its related

mechanisms, and the immune response. In sheep infected with louping-ill virus only low levels of interferon were present in the serum during the viraemic phase or in the brain homogenates from animals that succumbed (Vantsis, personal communication).

In numerous studies of the role of interferon in togavirus infections it is apparent that the levels of interferon both in the serum and the brain strictly follow the levels of virus found. Examples include Sindbis virus (Vilček, 1964; Schleupner, Postic, Armstrong, Atchison and Ho, 1969), West Nile virus (Subrahmanyan, 1968; Weiner, Cole and Nathanson, 1970), dengue virus (Cole and Wisseman, 1969b), tick-borne encephalitis virus (Vilček and Stanček, 1963; Hofmann, Radda and Pretzmann, 1971; Mayer, Mitrová, Gajdošová and Dakočil, 1974) and Langat virus (Thind and Price, 1969). The administration of interferon inducers provided some protection if given prior to infection or within the first 24 hours following infection with Japanese B encephalitis virus (Postic and Sather, 1970), tick-borne encephalitis virus (Hofmann, Kunz and Dippe, 1973), West Nile virus (Haahr, 1971) and dengue virus (Cole and Wisseman 1969b), but very little protection was induced after infection was established. Weiner et al (1970) concluded that interferon does not play an important role in recovery from virus infection of the CNS. Immunological mechanisms would therefore appear to be of greater importance. There are two lines of evidence to support this suggestion in the work reported here.

Following peripheral challenge of susceptible sheep viraemia was detected in the majority of cases within 24 hours. Thereafter the level of viraemia increased exponentially for the subsequent two to three days followed by a rapid decline. The decline was consistently associated with the appearance of both neutralizing and HI antibody. Several other togavirus infections behave likewise: dengue virus in mice (Cole and Wisseman, 1969a; Cole et al, 1973), Langat virus in mice (Webb, Wight, Wiernik, Platt and Smith, 1968b) Venezuelan equine encephalitis virus in mice (Kundin, 1966), hamsters (Austin and Scherer, 1971; Cole, Pedersen and Robinson, 1972) and monkeys (Gleiser, Gochenour, Berge and Tigertt, 1961) and tick-borne encephalitis virus in sheep (Málcová, 1960) and hamsters (Šimon, Slonim and Závadová, 1966; Slonim, Závadová and Šimon, 1966). That the immune response is of major importance in the cessation of viraemia is further indicated by studies where immuno-suppression has led to marked potentiation of viraemia with Langat virus (Webb et al, 1968b), Japanese B encephalitis virus (Thind and Price, 1969) and Kemerovo virus (Mayer, 1972).

In both sheep and grouse it was seen that the viraemias of the survivors were significantly lower and shorter than those detected in the susceptibles and antibody was consistently detected earlier in the serum of surviving sheep, which suggests a relationship between the magnitude and duration of viraemia, the rate of the serum antibody response and survival. It is, however, a feature

of togavirus infections that following the viraemic phase, virus persists in the CNS and the pathological processes leading to the development of clinical signs and death occur after this. Serum antibodies *per se* are unlikely to affect the course of virus replication in the extra-vascular tissues. Exchange between the CSF and interstitial fluid of the brain occurs via patent extracellular channels (Cserr, 1974); the CSF therefore accurately reflects the composition of the interstitial fluid of the brain. Antibody to louping-ill virus was detected in the CSF of all sheep following inoculation with louping-ill virus. In addition, in the acute phase of the disease much of the serum antibody of sheep was IgM. The molecular weight of human IgM is 900,000 (Cohen and Milstein, 1967) which normally excludes it from the CSF, and in the absence of a marked alteration in the permeability of the vascular-endothelium IgM will not enter the CSF from the blood stream (Heremans, 1968). In CSF collected from sheep that succumbed the total protein levels were only slightly higher than found in control CSF. Thus, serum IgM must be excluded from the CSF of sheep. The inability of the humoral antibody response to directly affect viral replication in the brain, particularly during the early stages of the disease, is therefore apparent. Hall (1969) in considering viral infections in general came to a similar conclusion with regard to the humoral immune response. Other mechanisms must therefore be involved.

The humoral immune response of sheep was seen to follow what

is now regarded as the normal sequence in a primary immune response. In every case the initial HI antibody was found to be IgM which was progressively replaced by IgG. This sequence of events has been observed in a number of species following inoculation with a variety of togaviruses (Table 6:2). The antibody response of sheep following initial experience with louping-ill virus therefore may be regarded as typical of a mammalian response to initial experience with a togavirus.

The CNS is an immunologically privileged site but this only holds true for antigens that are not experienced peripherally which was explained by the absence of a direct lymphatic drainage system from the CNS (Medawar , 1948). However, excellent evidence that an immune response may occur locally in the CNS following virus infection was produced by Morgan, (1947) in her studies of polio virus in monkeys and also by Schlesinger (1949) in studies of equine encephalitis virus in mice. It has now become increasingly evident that γ-globulin may be locally synthesized in the CNS in a wide variety of pathological conditions in man and Heremans (1968) in reviewing the subject listed neurosyphilis, tuberculous meningitis, African trypanosomiasis, chronic lymphocytic meningitis, selected cases of brain abscesses, bacterial meningo-encephalitis, neuromeningeal toxoplasmosis, subacute sclerosing panencephalitis, multiple sclerosis, intrathaecal lymphomatosis and a miscellaneous group of neurological disorders of unknown origin, as conditions where this apparently occurred.

Table 6:2. Togavirus infections in which the initial humoral antibody response has been found to be IgM.

Vertebrate	Virus	Reference
Man	Japanese B	Edelman and Poriyanonda,1973.
	Dengue	Russel, Intavivat and Konchanapilant,1969.
	Yellow Fever	Monath,1971.
	West Nile	Speir,1969.
	St. Louis	Speir,1969.
Gibbon	Dengue	Edelman Nisalak, Poriyanonda, Udomsakdi and Johnsen,1973.
Guinea Pig	Japanese B	
	Russian Summer Spring encephalitis	Bellanti, Russ, Holmes and Buescher,1965.
	Kunjin	Westaway,1968a.
Rabbit	Murray Valley encephalitis	Westaway,1968b.
	Kunjin	Westaway, Della-Porta and Reedman,1974.
	St. Louis	Westaway, Della-Porta and Reedman,1974.
	Japanese B	Westaway, Della-Porta and Reedman,1974.
	West Nile	Westaway, Della-Porta and Reedman,1974.
	Alfuy	Westaway, Della-Porta and Reedman,1974.
Mouse	Langat	Webb, Wight, Wiernik, Platt and Smith,1968b.
Calf	Murray Valley encephalitis	Sanderson,1968a, b.
	Getah	Sanderson,1968a, b.
Brown Bat	Japanese B	Leonard, Allen and Sulkin, 1968.

It is concluded from the studies reported here that sheep infected with louping-ill virus are capable of developing a local immune reaction in the CNS. This may be deduced from the fact that sheep infected with louping-ill developed relatively high titres of antibody in the CSF to the virus when compared to serum antibody to an antigen not involved in the disease process and from the fact that IgM was detected in the CSF of animals that succumbed.

In studies of antibody in CSF the relative titre to that of a serum antibody in rabbits was shown to be fairly constantly in the region of 1/300 (Freund, 1930). Similarly, in monkeys vaccinated with the Lansing strain of poliovirus Morgan (1949) found a ratio of between 1/250 and 1/1000. A similar ratio of antibody was also found in man to poliovirus (Clarke *et al*, 1965) and of iso-agglutinins (Lipton, Steigman and Dizon, 1965) as was antibody to egg albumen in rabbits (Sherwin, Richter, Cosgrove and Rose, 1963). The relative titre of antibody to egg albumen in CSF and serum of sheep injected with a vaccine prepared from infected egg embryos was also found to be of this order (Chapter 2).

The ratio of antibody to egg albumen in CSF and serum of sheep infected with louping-ill virus ranged from 1/80 to 1/1280. The low levels of serum antibody in some of the animals together with non-specific agglutination caused by CSF at low dilutions contributed to some animals apparently having a rather low ratio.

There was therefore no evidence of excess transudation of antibody into the CSF compartment either during the acute phase of disease or subsequently. The ratio of antibody to louping-ill virus was, however, lower; ranging from 1/5 to 1/20, both during the acute phase of infection and in animals that survived. Thus the ratio of antibody to an antigen not involved in the disease process was markedly higher than to viral antigen. This could not have arisen due to leakage of antibody from the vascular compartment hence other mechanisms must be involved. This was also shown to be the case in louping-ill virus infection of man (Webb et al, 1968a), where it was found that the ratio of antibody to polio-virus was considerably higher than the ratio to louping-ill virus. These authors suggest that this could only have arisen if at least some of the antibody present in the CSF was due to local production within the CNS.

Adopting this approach to investigating certain conditions in man the ratio of antibody in serum and CSF to a serum marker and to viral agents suspected to be present in the CNS has produced evidence that local production of antibody may occur in: subacute sclerosing panencephalitis (Conolly, 1968; Salmi, Norby and Panelius, 1972; Salmi, Panelius and Vainionpää, 1974; Winchester and Hambling, 1972), multiplesclerosis (Adams and Imagwa, 1962; Brown, Cathala, Gajdusek and Gibbs, 1971; Salmi, Panelius, Halonen, Rinne and Penttinen, 1972; Winchester and Hambling, 1972) acute measles encephalitis (Connolly, Haire and Hadden, 1971), encephalitis associated with infectious mononucleosis (Epstein

Barr virus) and herpes simplex virus (Joncas, Chicoine, Thivierge and Bertrand, 1974) and polio virus (Lipton et al, 1965; Ogra, Ogra, Al-Nakeeb and Coppola, 1973). In these cases the conclusion that at least some of the antibody activity detected in the CSF was locally synthesized appeared inescapable.

The relatively higher total protein levels recorded in the animals that became moribund may have been due to cell elements present in the CSF. No attempt was made to clarify the CSF or to detect cells prior to storage in this study but raised cell counts have been found in the CSF of man (Rivers and Schwentker, 1934; Brewis, Neubauer and Hurst, 1949; Webb et al, 1968a) monkeys (Galloway and Perdrau, 1935) and cattle (Dunn, 1952) infected with louping-ill virus. The absence of raised levels of antibody to egg albumen in the CSF from the acute cases make it unlikely that the increased levels of protein detected arose from increased vascular permeability.

IgM was invariably present in CSF of sheep that succumbed but could not be detected in CSF from any of the survivors nor in CSF from controls. The presence of IgM in CSF has also been detected in certain pathological conditions of man. In human trypanosomiasis IgM has been detected in CSF but ^{125}I labelled IgM administered intravenously did not enter the CSF of such cases (Masseyeff, Blondel and Mattern, 1972; Greenwood and Whittle, 1973). Cells positive for IgM using fluorescent antibody techniques were detected in the CSF of such patients. IgM has

also been detected in CSF of patients with subacute sclerosing panencephalitis (Connolly et al, 1971) and a patient with chronic progressive encephalopathy who possessed no serum IgM (Lord, Dupree, Goldblum, Storey, Forman and Goldman, 1973). CSF from cases of pneumococcal meningitis (O'Toole, Thornton, Mukherjee and Neogy, 1971) and meningo-encephalitis due to Angiostrongylus cantonensis (Tungkanak, Sirisinha and Punyagupta, 1972) have also contained IgM. In addition cells containing IgM have been identified in CSF collected from patients within the first week after the onset of viral encephalitis (Dayan and Stokes, 1973). Finally, the CSF collected at autopsy from seven out of 16 cases of canine distemper encephalitis contained IgM (Cutler and Averill, 1969).

In all these instances it was concluded that IgM was present due to local synthesis. In immunofluorescent studies of the perivascular cuff of sheep which became moribund following inoculation with louping-ill virus IgM staining cells were present (Doherty, Reid and Smith, 1971). It would seem reasonable to conclude that these cells were the origin of the IgM detected in the CSF of these sheep.

The HI activity detected in CSF collected from sheep between the 7th and 10th days was reduced by between 16- and 64-fold by 2-ME treatment. In addition, when CSF from such cases was passed through a Sephadex G-200 column all the activity was associated with the first exclusion peak. Much of the specific viral

antibody is seen to be associated with the IgM class of antibody during the acute phase of infection. CSF collected from the surviving animals, on the other hand, possessed HI antibody which appeared to be entirely IgG.

The dynamics of the appearance of antibody in the CSF and serum therefore followed the same pattern. The early appearance of antibody in the serum was associated with survival. Thus it would seem reasonable to conclude that in such cases the local immune response in the CNS was also activated earlier. The early invasion of cells capable of releasing antibody within the CNS therefore appears to be critical in determining the outcome of infection. It may, therefore, be concluded that in cases that recover antibody is capable of limiting the spread of virus and the quantitative involvement of neurons, resulting in a sub-clinical infection, in animals that succumb the degree of neuronal damage is sufficiently extensive by the time antibody is released within the CNS that clinical louping-ill results. The variable outcome of infection with louping-ill virus therefore appears to be determined by the rapidity with which the immune reaction is activated.

CHAPTER 7

DISCUSSION OF THE EPIDEMIOLOGY OF LOUPING-ILL

The epidemiological implication of this work cannot be discussed in isolation from the vector. It is, therefore, necessary to summarise the relevant features of the biology of I. ricinus. The life cycle of I. ricinus from egg to engorged adult takes a period of three years during which no more than three weeks are spent on a vertebrate host (Lees and Milne, 1951). It is therefore self evident that the distribution of I. ricinus will be determined by the availability of a suitable micro-habitat conducive to survival away from the host, the essential pre-requisite of which is a relative humidity close to saturation (MacLeod, 1934b, 1935). The structure of the vegetation and the nature of the soil over much of the hill grazing of Scotland ensures that the humidity of the layer close to the soil provides such a micro-climate throughout the year (MacLeod, 1936; Milne, 1944, 1950a), and MacLeod (1932) found that sheep throughout much of the hill pastures of Scotland were parasitized. The factors controlling the level of infestation of an area are less clear but in balance it can be concluded that it is governed primarily by the host potential. Milne (1949b) lists 29 mammals and 39 birds on which at least one stage of I. ricinus has been found. The list is almost certainly incomplete, as probably any vertebrate above amphibia which either feeds or nests on the

ground can be parasitized (Arthur, 1963). The host range of the adult tick is, however, restricted to the larger species and probably only mammals supply a significant number of blood meals to this stage (Milne, 1949a).

To ascertain accurately the relative importance of a vertebrate in the maintenance of I. ricinus information on the levels of parasitism and of the comparative abundance of the species are required. No such study has, as yet, been undertaken but from the available evidence certain tentative conclusions may be made. The levels of infestation found on wild vertebrate hosts collected in the North of England (Milne, 1949b) and in Ayrshire (Varma and Smith, 1971) compared with those on sheep were quite insignificant and Milne (1949b) concluded that between 94-99% of the tick population was supported by sheep. This conclusion would appear to confirm the observation of MacLeod (1934a) in the South of Scotland who recorded a dramatic reduction of the tick population in a ten acre plot from which sheep had been excluded for a period of 18 months.

Voles have been considered of major importance in the population dynamics of I. ricinus in Czechoslovakia (Rosický and Černý, 1954). In Scotland, however, the vole population is at its lowest during the period of maximum tick activity, which does not suggest that a similar situation exists here. In assessing the role of small mammals in the maintenance of I. ricinus the observation of Milne (1950b) may also be pertinent. He found

that over 50% of the engorged female ticks returned to a sheep pasture were killed or removed by predators prior to oviposition and produced evidence to suggest that the shrew (Sorex araneus) was principally involved.

The red deer (Cervus elaphus) has been considered as potentially important in the maintenance of I. ricinus in Scotland (Moore, 1938). However, their low numbers, grazing and behavioural pattern makes it unlikely except in very limited areas (MacLeod, 1938).

In certain situations the maintenance of I. ricinus may to some extent depend on the blue hare (Lepus timidus). Moore (1937) found that hares were "heavily" parasitised and a control programme involving the shooting of 3044 hares greatly reduced the degree of infestation found on sheep. This would be in agreement with a current investigation where, in some areas of Speyside, evidence is emerging that hares may be the principal maintenance host. In both these situations the hare population would appear to be unusually high and perhaps should be regarded as atypical.

Cattle and sheep are the only domestic animals present in appreciable numbers in the hill farming areas. The total hill cattle population is little in excess of 300,000 whereas the total breeding sheep stock is in the region of 2½ million (Cunningham, Smith and Doney, 1970). On numerical grounds alone when compared to sheep, cattle can only play a minor role. Furthermore,

cattle stocking tends to be restricted to the more fertile areas where it is less likely that the micro-climate essential for tick survival will be maintained. The conclusion of MacLeod (1932) that in Scotland sheep were by far the most important host would, therefore, appear to be generally true. It is, therefore, apparent that in areas where the nature of the vegetation and soil provide a suitable micro-climate for the survival of ticks the principal factor governing the degree of infestation will be the sheep stocking rate.

An additional feature of the biology of I. ricinus which requires consideration is the annual periodicity which has been described by all investigators. In general, throughout Scotland there is a period of intense activity during the spring which is followed by a period of relative quiescence throughout the summer. This is followed in the west of the country by an autumn recrudescence of less intensity which occurs from late August to October (MacLeod, 1940; Lees and Milne, 1951).

The controlling factor would appear to be temperature; in spring prior to the weekly average daily maximum temperature exceeding 7° C MacLeod (1936) found that sheep were virtually free of ticks. The lower limit at which ticks in the laboratory became active was 11° C (MacLeod, 1935). However, if allowance is made for the fact that the average weekly temperature recorded the air temperature in the shade whereas the tick inhabits a micro-climate close to the soil there is, in fact, reasonable

correlation between these figures.

During a period of eight years MacLeod (1940) found in Argyllshire the peak activity was in early May, mid-May in Perthshire and at the end of May in the Borders. This pattern of activity was later confirmed in Northumberland (Milne, 1947) and in Wales (Edwards and Arthur, 1947). In all areas the period of peak infestation was followed by a rapid decline to comparatively low levels by from early to mid-June. This decline was attributed by MacLeod (1936, 1940) to the average weekly maximum temperature exceeding 16° C. However, experiments conducted subsequently (Lees and Milne (1951) leave little doubt that the reason is firstly depletion due to ticks successfully attaching to a host and secondly to natural mortality as the average period of activity of a tick under natural conditions was 30 days, of which only nine days were spent at the vegetation tips actively questing.

In summary it would appear that the spring activity commences when the air temperatures reach a threshold level of 7° C. Ticks failing to gain a host during the subsequent 30 days die due to desiccation and exhaustion of their fat stores (Lees and Milne, 1951; Campbell, 1952). Larvae and nymphs that engorge in the spring undergo metamorphosis during the summer months while the females lay eggs. Egg hatching and moulting is not however complete until the end of October and the emerging stages can seldom be persuaded to feed before December (Campbell, 1952). The autumn recrudescence of ticks therefore represents a separate

population. Ticks that engorge in the autumn do not complete metamorphosis until the following July and similarly the eggs that result from autumn fed females do not emerge until this time. Such ticks become active in the autumn and if they fail to gain access to a host may survive the winter and become active the following spring. Thus although there can be no exchange of the spring active population into the autumn population some autumn active ticks may join the spring active population (Lees and Milne, 1951; Campbell, 1952). However, for the purposes of this discussion they will be regarded as separate populations.

An exception to the general pattern of activity was reported by Moore (1938) on one grazing in the north east of Scotland where the peak activity was in July. This pattern was subsequently reported from six other farms in the north east (Arthur, 1963) and in Speyside (Duncan, personal communication). These observations have, however, been made on habitats which are over 800 to 1000 feet above sea level, where the air temperature in the spring may not reach the threshold for tick activity until later in the year. It is therefore probable that the factors which determine the periodicity of tick activity throughout Scotland are the same.

From the above analysis the prevalence of the vector of louping-ill is seen to depend largely on the availability of sheep. The flock structure and system of management must therefore be considered in relation to I. ricinus. Generally

lambing occurs from mid-April to May. Lambs are reared on the hill grazing until mid-August to October, when the wethers and surplus hoggs are sold. The hoggs to be retained are either brought to low ground for wintering or sent to lowland arable farms. Hoggs are then returned to the hill grazing in the following spring where they are retained until sold at between five to six years of age. Although the mortality experienced amongst ewes and the age at which ewes are sold varies considerably on different farms and in different parts of the country (Cmd. 6494, 1944) this management regime is generally representative.

Lambs are born at or about the time of maximum tick activity. However the very young lamb due to its comparatively small size and physical inactivity does not encounter questing ticks to the same extent as adults. From observations in the north of England it was calculated that during the spring active period a ewe would be parasitized by approximately eight times more ticks than would a lamb (Milne, 1949b). The practice of removing lambs from the hill pasture towards the end of August also tends to prevent lambs from contacting questing ticks in the Autumn active period. Despite the effective doubling of the sheep population during the summer months due to the presence of lambs their influence on the host potential of an area will probably only be minor. By inference, therefore, the host potential of an area will be determined by the resident breeding stock; ewe lambs not being heavily parasitized until returned to the hills when approximately

one year old.

The epidemiology of louping-ill will be determined by the interaction of the vector with the vertebrate host population. It is apparent from the above discussion that the sheep is by far the most important host for the tick, and this aspect will be considered first. Other possible cycles will be considered later.

From the work described here on the pathogenesis of infection in sheep certain conclusions may be made with regard to their role in the maintenance of louping-ill virus. Following infection by subcutaneous inoculation which may be regarded as analogous to introduction of virus by ticks a viraemia develops. In the majority of cases the viraemia reaches levels exceeding 10^2 mouse ID_{50} per 0.03 ml; the threshold of infection for the tick (Swanepoel, 1968). This was true both in three-day-old and nine-month-old animals. For the purpose of this discussion, therefore, all infections of susceptible sheep will be regarded as being potentially infective to ticks. The cessation of viraemia was associated with the development of serum antibody and this occurred in all cases prior to death. The outcome of infection does not influence the capacity of the sheep to infect ticks; the period between the cessation of viraemia and death was in general sufficient to permit ticks feeding during the viraemic phase to become replete prior to death intervening. Serum antibody that develops following infection is maintained for at least three years and probably for life; an individual

animal can undergo only one viraemic phase. In addition such antibody is effectively transferred in the colostrum, the levels of antibody achieved by lambs being of the same order as that of their dams (Wilson and Gordon, 1948; Williams and Thorburn, 1961; O'Reilly, Smith, McMahon, Bowen and White, 1968; Brotherston et al, 1971). Lambs that acquired maternal antibody were initially completely refractory to infection which is in accordance with the results of Wilson and Gordon (1948) and Williams and Thorburn (1961). The half-life of this antibody has been calculated to be 13.7 days (Brotherston, personal communication). When the levels of serum HI antibody in lambs decline to 1/20 active immunity may develop in the absence of viraemia and this state is maintained for approximately two months. Following this period lambs are susceptible.

In order to assess the effect that these features of infection will have on the capacity of sheep to maintain louping-ill virus a flock consisting of x breeding animals will be considered. Apart from the replacement hoggs which will be discussed later such a flock will consist during the spring tick active period of both immune (y) and non-immune animals; the number of susceptible sheep will be x - y. There is no reason why the breeding success of immune animals should differ appreciably from non-immune, then, provided there is efficient transfer of colostrum, there will also be x - y susceptible lambs, assuming the overall lambing rate to be unity. It can be anticipated that throughout the spring active period the levels of maternal antibody present

in lambs would be sufficient to render them totally refractory to infection.

From the work of Milne discussed above lambs will only be parasitized by 1/8th of the ticks that infest the adult flock. As it may be assumed that infective ticks are dispersed randomly in the tick population the probability of a lamb encountering infection is only 1/8th that of an adult. The potential importance of infection in lambs is further reduced by the fact that during the viraemic phase it may be anticipated that a lamb will be parasitized by only 1/8th of the ticks that would be present on an adult during this phase. The number of ticks infected by the adult flock will be a function of $x - y$, while in the lamb population it will be a function of $\frac{x - y}{64}$. Provided there is efficient transfer of maternal antibody the role of lambs in the maintenance of louping-ill in the spring tick population can only be minor and this will hold true irrespective of the rate of infection.

During subsequent months antibody levels in the lambs will decline while physical size and activity will increase. In areas where there is an autumn recrudescence of ticks not only will the lambs have a host potential for ticks approaching that of the adult but the immune status to louping-ill of the population will have altered. The susceptible lambs will now consist of those that did not receive maternal antibody together with a proportion of the lambs in which maternal protection has completely waned.

The levels of antibody present in the majority of the remainder will probably have dropped to a level at which infection may occur in the absence of viraemia. The relative proportion in each group will depend both on the level of antibody transferred and the timing of the autumn recrudescence. The point in time relative to the tick activity at which wether lambs and surplus hoggs are removed for sale will also influence the potential of the lambs to infect ticks. The autumn tick rise is generally of less intensity than that of the spring and infections associated with this period of activity are less frequently reported. Lambs, however, may play a greater part in the maintenance of virus in this population of ticks, which would be particularly true where the level of infection in the spring was high, rendering virtually all the adult flock immune.

Dependent on the prevailing form of management replacement stock are sent for winter grazing on lowland farms or are kept on the hill pasture. It is unlikely that either practice will influence the maintenance of virus as during this period tick activity is minimal.

In the succeeding spring irrespective of the form of management adopted the immune status of the hoggs will be the same. Maternal antibody will have completely disappeared and only those that have experienced active infection in the previous spring or autumn will be immune. Very few of these animals will have been infected as lambs in the spring or as hoggs in the

autumn. The hoggs will therefore in general present a totally susceptible population, and numerically they will represent approximately 1/5th of the breeding flock. The degree of tick infestation of hoggs was found to be of the same order as of ewes (Milne, 1949b), hence the probability of a hogg encountering infection is the same as that of a ewe. When allowance is made for the proportion of ticks feeding on lambs and adult ewes, the hoggs can be calculated to provide a blood meal for approximately 1/6th of the total tick population parasitizing the flock. Hence the proportion of ticks feeding on virus susceptible hosts is unlikely to fall below 1/6th.

Milne (1947) calculated that 1/5 th of the total tick population that succeeded in gaining a host was present at the peak of activity. As the degree of parasitism will be directly related to the probability of encountering infection it may be assumed that on average 1/10th of the ticks parasitizing a susceptible animal that becomes infected will do so while the animal is viraemic. If it is assumed that all the hoggs become infected it may be deduced that 1/60th of the ticks will derive their blood meal from a viraemic host. However the proportion of ticks that become infected following feeding on a viraemic host has never been found to be greater than 10% (Beesley, personal communication). Hence the proportion of ticks that can be anticipated to be infected the following spring would be 1/600. The total number of ticks that parasitize on an individual sheep during the spring

active season in different parts of Scotland and the north of England was calculated to be greatly in excess of 600 (MacLeod, 1940; Milne, 1949b). Under normal conditions of farm management it can, therefore, be concluded that the sheep flock would be able to maintain louping-ill virus and there would be a tendency for all animals to experience infection as hoggs. There can be little doubt, therefore, that sheep alone would be capable of maintaining louping-ill virus and that under most circumstances this would depend largely on the hoggs.

The limited reports of naturally occurring infection would seem in general to support this conclusion. The majority of such reports are based entirely on the clinical occurrence of infection and their interpretation must be cautious. The classic situation appears to be that the principal mortality experienced is in the hogg population on return from winter pasture and disease in the adult flock is a rare occurrence (Poole et al, 1930; Gordon et al, 1932a,1962; Williams and Thorburn, 1962; Smith et al, 1964; Brotherston et al, 1971). This would infer that, in general, there was a relatively high rate of challenge and that few animals escaped infection in the first year. However, in addition to mortality in the hoggs considerable mortality due to louping-ill in lambs has been reported (Gordon, 1934; Smith et al, 1964) in one farm reaching 38% and on another occasion 50%. In the absence of any mention of mortality in the breeding flock it must be presumed that this was not striking; these losses apparently

occurred in flocks where the proportion of immune animals was high. Indeed, the problem was considered of sufficient magnitude to warrant an extensive field trial employing immune serum involving approximately 20,000 lambs and 15,000 controls (Wilson and Gordon, 1948). These claims however may be regarded with some scepticism as during a subsequent investigation which Smith et al (1964) conducted they succeeded in isolating virus from only one of eight lambs submitted for virological investigations. This would appear to confirm the opinion of M'Gowan and Rettie (1913) that 95% of cases of "louping-ill" in lambs were due to "navel or joint-ill".

Losses due to louping-ill virus in lambs in the absence of disease in the adult sheep could, however, be explained if there was failure to transfer maternal antibody. In investigations of mortality among hill lambs, γ-globulin has been found to be absent or present in negligible levels in a proportion (Haliday, 1965; Reid, 1972). Similarly findings were reported by Harker (1974) in housed lambs. The efficient transfer of louping-ill virus antibody in the colostrum found by Williams and Thorburn (1961); O'Reilly, Smith, McMahon, Bowen and White (1968) and Brotherston et al (1971) in lambs kept under laboratory conditions may not occur to the same extent under commercial conditions of management. It is, however, evident that lambs that do not have detectable levels of γ-globulin are less likely to survive the first ten days of life (Halliday, 1965; Findlay, 1973 and Harker,

1974). Only a proportion of such lambs could therefore become infected with louping-ill virus. All losses are, however, liable to be attributed to louping-ill if cases subsequently occur. Support to this view that failure of transfer of maternal antibody may contribute to losses is provided in the field trial of Wilson and Gordon (1948). Lambs that did not receive immune serum experienced a 13% mortality as opposed to 6.5% in those that received horse anti-louping-ill serum and 3.8% of those that received sheep anti-serum. They regard these results as only partially due to the protection provided against louping-ill virus infection the rest being attributed to the "at present an immeasurable benefit of serum". This "immeasurable benefit" may have been due to the effect of administering serum to lambs, a proportion of which were hypo- γ -globulinaemic due to failure to absorb sufficient maternal antibody, which would provide protection against a variety of conditions other than louping-ill (Piercy, 1974).

It is, therefore, felt that reports of mortality experienced in lambs due to louping-ill cannot be regarded as indicative of infection. The role of such infections in the maintenance of the virus cannot be ascertained at the present time. It is, however, unlikely to be of the same order as that of hoggs due to the lower levels of parasitism which will be associated with infection in lambs.

Attempts to identify virus in ticks infesting pastures have

been negligible. Smith (unpublished cited by Varma and Smith, 1972) estimated that the infection rate of ticks on a study area in Ayrshire was 1/600 but no details of the numbers examined or methods employed are provided and Swanepoel (1968) recovered a single isolate from 1284 nymphs collected in Argyllshire. These observations are compatible with the calculated infection rates that would be anticipated if the hoggs were primarily responsible for the maintenance of louping-ill virus.

From the above considerations it is apparent that throughout much of Scotland louping-ill virus could be maintained in a sheep-tick cycle. The hoggs being almost entirely responsible for the infection of ticks. By inference therefore effective vaccination of the hoggs prior to return to the hill could effectively eliminate the sheep from this cycle. Prior to the introduction of a tissue culture vaccine which stimulated a serum antibody response (Brotherston and Boyce, 1969, 1970) inactivated brain vaccine had been used. It was claimed, that although such vaccine failed to induce the production of serum antibody to louping-ill virus, it sensitized sheep to viral antigen (Gordon, 1934; Gordon, Brownlee, Wilson and MacLeod, 1962; Edwards, 1947; Smith et al, 1964; Smith, 1969; Brotherston et al, 1971). Sensitized sheep were suggested to develop an active immunity when challenged in the absence of clinical disease. Although no critical laboratory tests were conducted to confirm this hypothesis its application in the field did appear to reduce losses (Gordon, 1934 and Gordon

et al, 1962). From the present studies it may be concluded that animals sensitized to the viral antigen would develop a more rapid immune response and would therefore be protected from clinical louping-ill. Sensitized animals are, however, unlikely to develop a viraemia of a magnitude sufficient to infect ticks. Hence the systematic inoculation of hoggs with such a vaccine would be expected to prevent these animals from transmitting virus to ticks. If such was the case, from the present analysis, louping-ill virus could be anticipated to be eliminated from the tick population of a hill grazing.

The validity of this conclusion cannot at the present time be ascertained. It is, however, of interest that whereas in the 1930's louping-ill was of great concern to the Border sheep farmers it no longer appears to be so, to the same extent. It is unlikely that any dipping regime considered practicable for sheep would have led to any appreciable degree of reduction of the tick population. Hence this apparent reduction in the incidence of louping-ill must be either due to the effect of the vaccine or other factors as yet undefined.

From the above discussion it is apparent that in areas where sheep are grazed the bulk of the tick population is maintained by sheep and in the absence of systematic vaccination louping-ill virus can be perpetuated in an exclusively sheep-tick cycle. There is, however, limited evidence of naturally occurring infection in several feral species. Virus has been isolated from

two of 26 wood mice and one of 68 common shrews examined (Smith, Varma and McMahon, 1964) and antibodies have been detected in the sera of red deer (Dunn, 1960). Recent serological studies have indicated that infection of the European badger (Meles meles), blue hare, rabbit (Oryctolagus cuniculus) and roe deer (Capreolus capreolus) also occurred (Reid, unpublished). In the absence of experimental studies to determine the intensity of viraemia that develops in these species it is impossible to ascertain the significance of these observations. The isolation from the shrew is of interest in view of the predation of this species on adult engorged ticks (Milne, 1950b) and that only 26 of 363 (7%) shrews were found infested with ticks (Varma and Smith, 1971). This suggests that the shrew could acquire infection by ingestion of the infected ticks rather than by tick bite.

Published data of experimental infection of captive feral animals is even more limited. The only species in which the viraemia has been systematically studied is the vole (Microtus agrestis) (Seamer and Zlotnik, 1970). These authors conclude that the intensity of viraemia that developed following peripheral inoculation was unlikely to infect I. ricinus. Studies of laboratory mice, hamsters and guinea pigs also indicate that the levels of louping-ill viraemia that develop in these species are low when compared to other members of the tick-borne encephalitis group (Pogodina, 1964; Pogodina and Savinov, 1964; Doherty, 1969a, b). In addition unpublished work conducted at Moredun Institute indicates that the red fox (Vulpes vulpes), wood mouse

and brown rat (Rattus norvegicus) are unlikely to develop viraemias of a magnitude sufficient to infect the tick (Stevenson, personal communication). The limited evidence available suggests no role for naturally occurring Mammalia in the maintenance of louping-ill virus. Further studies are clearly indicated, in particular, of those species which have been shown on the basis of serology to be regularly infected under natural conditions, mainly red deer and blue hare.

At the present time therefore the only wild species that has been identified capable of developing a viraemia sufficient to infect I. ricinus is the red grouse. A total of 11 isolates of virus has been achieved from this species (Williams et al, 1963; Watt et al, 1963; Reid and Boyce, 1974). In addition Williams et al (1963) investigated a number of healthy birds for antibodies, but these authors apparently omitted to extract the sera prior to testing for HI activity; the results cannot be relied on. Reid and Boyce (1974) found HI activity in four out of 125 extracted grouse sera at titres of between 1/1280 and 1/2560 suggesting that this activity was due to antibody. On the basis therefore of both virus isolation and serology, infection of red grouse may be concluded to occur naturally. Recent studies indicate that this may be frequent in some areas (Duncan, personal communication). Duncan has found that the survival of grouse in Speyside is related to the prevalence of ticks as assessed by both blanket drags and counts of engorging ticks on young grouse.

In areas of high tick infestation few or no birds are reared and all the surviving birds possess antibodies while in areas of low infestation the average brood consists of seven young birds none of which possess antibody to louping-ill virus. There is therefore growing evidence that louping-ill virus infection may be a serious cause of mortality in free living populations of grouse in some areas.

Williams et al (1963) infected two birds both of which died on day seven and eight post-inoculation. However, no attempt was made to assess the intensity of viraemia nor was nerve cell damage detected on histopathological examination. It was not, therefore, possible to assess the significance of their results. The experimental studies described here confirm and extend these observations. All birds that were infected with virus became clinically affected and 79% died. In addition virus was isolated from the brains of the eight birds that survived and were killed on the 14th and 19th days respectively and severe lesions were detected histologically (Buxton and Reid, 1975). Although these birds had apparently recovered from infection the persistence of virus and presence of severe lesions following the acute phase of the disease, leaves some doubt as to whether they would have survived under natural conditions. These experimental studies therefore confirm the suggestion that louping-ill virus infection of free-living birds may frequently have a fatal outcome.

Mortality amongst avian species has been attributed to

infection with other togaviruses. A proportion of pheasants (Phasianus colchicus) and pigeons (Columba livia) infected with members of the equine encephalitis virus group have been reported to die (Fothergill and Dingle, 1938; Tyzzer, Sellars and Bennett, 1938; Kaplan, Winn and Palmer, 1955; Luginbuhl, Satriano, Helmboldt, Lamson and Jungherr, 1958; Faddoul and Fellows, 1965). Both these species, however, have been introduced recently to the United States of America and the indigenous bird population probably experienced inapparent infection (Satriano, Luginbuhl, Wallis, Jungherr and Williamson, 1958; Karstad, Spalatin and Hanson, 1959). There are numerous reports of infection of avian species with a wide variety of togaviruses other than tick-borne encephalitis and equine encephalitis groups of viruses (e.g. Whitehead, Doherty, Domrow, Standfast and Wetters, 1968; Schmidt and Shope, 1971; Erneck, Kožuch, Sekeyóvá, Hudec and Folk, 1971; Erneck, Kožuch, Grešíková, Nosek and Sekeyóvá, 1973; Gaidamovich, Nikiforov, Gromashevsky, Obukhova, Klisenko, Chervonsky and Melnikova, 1971; Gaidsmovich, Kiryushchenko, Pierkre and Vasilenko, 1973; Dickerman, Scherer, Moorhouse, Toaz, Essex and Steele, 1972; Hore, Campbell and Turner, 1972; Saikku and Brummer-Korvenkontia, 1973). Evidence that tick-borne encephalitis virus also infects birds has been reported including a variety of passerines (Fedorov, 1958; Ernek et al, 1971) coots (Fulica atra) (Sobeslavský, Rehn and Fischer, 1960) and wild duck (Chagula hyemalis) and (Melanitta fusca) (Pogodina,1962). However, except for the last cited report no suggestion of mortality

associated with infection is made. In this report it is claimed that "about 1000 birds died" and subsequently virus isolation was achieved from four apparently healthy birds that were shot. This cannot be accepted as evidence of mortality due to infection with tick-borne encephalitis virus. Free-living birds would, therefore, appear to become infected with a variety of togaviruses but do not normally experience mortality following infection.

It is assumed that long association with the particular virus or viruses has led to the "elimination of the more vulnerable individuals and survival of those more tolerant to infection" (Blaškovič and Nosek, 1972) leading to the evolution of a virus-host relationship which may be regarded as "commensalism" (Blaškovič, 1962). No such relationship has developed in the case of louping-ill virus infection of grouse which suggests that this association may be of comparative recent origin.

The red grouse is probably the only avian species that naturally occurs exclusively in the British Isles (Leslie and Shipley, 1912). In addition antigenic analysis of the tick-borne encephalitis group suggests that louping-ill virus represents a sub-type unique to the British Isles (Clarke, 1964). It can therefore be concluded that both the virus and the host have been confined to the same geographical area for a very considerable period. The marked susceptibility of grouse to infection with louping-ill virus cannot be explained in terms of the recent introduction of either to the British Isles. It is, however, possible that the distribution of either the grouse or the virus may have altered so that contact has only occurred recently.

The grouse is, and probably always has been, associated with moorland where heather (Caluna vulgaris) is the dominant plant species and the distribution of such moorland has remained essentially the same for many centuries. It is therefore probable that grouse have always inhabited the same areas in which it is found today. The density of the grouse population may, however, have increased as management of moors for the purpose of increasing stocking rates has probably only been practised since the start of the 19th century (Douglas-Home, 1938). It is unlikely, however, that increased numbers alone could be responsible for the development of new foci of virus.

Land utilisation in Scotland has changed very markedly during the last two centuries. The following account is based on the report of the Committee on Hill Sheep Farming in Scotland (Cmd. 6494, 1944). Sheep farming in the hill districts of the southern counties has been practised for many centuries but north of the Forth and Clyde it is of comparative recent introduction. Before the introduction of sheep on a large scale relatively intensive subsistence farming had been practised with much of the low lying ground being under cultivation. The hill pasture was only utilised for a few months during the summer at which time both cattle and sheep were grazed in approximately equal numbers and the stocking rate was considered light.

From the end of the 18th century this type of farm practice was progressively replaced by large scale sheep farming until

around 1870 the industry was regarded to have reached its climax when the average total of sheep in the Counties of Argyll, Inverness, Ross and Cromarty, Sutherland and Perth was in excess of three million as opposed to an approximate 1.2 million in 1967 (Cunningham et al, 1970). This was followed by a period of gradual but progressive decline due in part to outside economic pressures but also the Committee conclude to a loss in the fertility of the land. The decline in fertility from the time when relatively intensive agriculture was practised is attributed both to the selective grazing habits of sheep and a lack of attention to drainage. They regard the increase in the ranker types of vegetation, coarse grass, heather and bracken and excessive moisture due to neglect of drains to have been instrumental in the increase of tick-borne diseases in the sheep population.

This conclusion is of interest but cannot be accepted. The micro-climatic requirements of the tick have probably always been maintained on much of the hill grazing; there may, however, have been encroachment of the ticks on to the areas previously devoted to crop cultivation. The area suitable for the survival of the tick may have extended but this is unlikely to have had an appreciable effect on the overall distribution of I. ricinus.

It was concluded earlier that the abundance of ticks in an area was related to the host potential. From the above historical account the host potential of domestic animals is seen to have

changed markedly during the last century. Prior to the introduction of sheep the stocking of the hill grazing is described as light and only occurred during the summer months. Not only was there comparatively fewer animals present but they may not have been placed on the hill until after the principal period of tick activity. The availability of domestic animals as hosts to the tick must therefore have been greatly increased by the introduction of large scale sheep farming as not only would there be heavier stocking rates but the animals would be available throughout the period of tick activity. The changes in agricultural practice which have occurred within the last two hundred years would appear to have greatly increased the host potential of the hill grazing in the areas north of the Forth and Clyde.

In addition sheep and cattle were present on the hill grazing in approximately equal numbers. Due to the irregular low viraemia detected in cattle following peripheral inoculation (Dunn, 1952) it is unlikely that infection of cattle would regularly be able to infect ticks. It is therefore concluded that the introduction of large scale sheep farming into the areas where grouse were present, will have led not only to a marked increase in the abundance of ticks, but also to the proportion of virus infected ticks.

Support for this concept is found in an early report of sheep diseases. Stevenson (1807) describes a clinical syndrome which at least in part is almost certainly due to louping-ill virus

infection. This condition was apparently not infrequently observed in the south but was "scarcely known in the Highlands". Although this was written 70 years before the zenith of the sheep industry in the Highland Region from this report it can be gathered that sheep by 1807 were extensively present in this area yet apparently in the absence of louping-ill. It is therefore possible that grouse prior to this only infrequently encountered infection thus there was no evolutionary pressure in favour of resistance to infection.

Although this conclusion based on historical appraisal must remain tentative it is not entirely without precedent. Accompanying the outbreak of Kyasanur Forest disease virus infection in man in the Sagar and Sorab forests of Mysore State, South India heavy mortality was observed in indigenous monkeys (Presbytis entellus)and(Macaca radiata) (Work, 1958). The circumstances surrounding the appearance of disease in man and monkeys were traced by Bøshell (1969). In the period prior to the outbreak of disease there had been a progressive increase in the human population of the area, which had led to encroachment of the forest areas for the purpose of grazing cattle. The tick involved in transmitting infection to both man and monkeys was Haemophysalis spinigera which depended almost entirely on cattle for maintenance. Cattle, however, developed either no viraemia or a transient viraemia of low intensity which was unlikely to infect ticks (Anderson and Singh, 1973). The introduction of cattle into the

forest areas contributed to a very marked increase in the abundance of H. spinigera but not to the infection of ticks. It was the increase in the tick population which apparently brought the monkeys into contact with virus for the first time. Previously virus had probably circulated as a hidden enzootic process in forest rodents. Introduction of virus into the monkey population led to their progressive decimation and an increase in the rate of infection of the ticks which, in turn, gave rise to infection in man.

The introduction of sheep in large numbers to the heather moors probably had a very similar effect on the I. ricinus population as cattle had on the H. spinigera population in forest areas of Mysore State in India. However, in addition sheep would have been able to infect ticks with virus. Prior to this the host potential of these areas for I. ricinus would have been meagre and from our existing knowledge it is doubtful if louping-ill virus could have been maintained. The introduction of a host capable of supporting a much higher population of ticks and of infecting ticks could therefore have effectively introduced virus to these areas for the first time.

It is, therefore, concluded that louping-ill virus may have first come in contact with the grouse population due to the effects of introducing sheep to the heather moors in large numbers. Whether grouse are capable of maintaining louping-ill virus in the absence of sheep cannot yet be definitely ascertained. Grouse

are seldom parasitized by the adult stage of I. ricinus (Milne, 1949a; Arthur, 1963; Duncan, personal communication); grouse alone are incapable of supporting the tick population. Alternative hosts therefore determine the abundance of ticks on the moors and grouse can only provide blood meals for a proportion of the immature stages. In the absence of information on the relative abundance of ticks on grouse and alternative hosts it is not possible, therefore, to assess the contribution that grouse will make to the maintenance of virus.

Virus may, however, influence the grouse population. It is emerging from current investigations that in areas where ticks are numerous, broods of normal size hatch but very few or no young birds survive. From the experimental studies and from attempts to isolate virus from sick and dead birds it is probable that louping-ill virus is involved in this mortality. Mortality in grouse has been observed for many years. In 1873 Mr. MacDonald reported to the Times with regard to grouse "it seems that a disease of an exceeding virulent nature prevails in all parts of the Highlands and in a form hitherto unknown ...". It would be highly presumptious to suggest this mortality could be attributed to louping-ill but it may be pertinent that the date of this report coincides with the period when the hill sheep industry was at its maximum. The problem of the "grouse disease" was sufficient that in 1905 the Board of Agriculture and Fisheries appointed a Committee to investigate the problem. This

Committee concluded in the absence of any concrete evidence that the principal cause of mortality was parasitism with Trichostrongylus pergracilis and Eimeria avium (Leslie and Shipley, 1912). They do, however, record that mortality could occur in all age groups and that in many areas there was a "mysterious disappearance of chicks". Both these features have now been associated with louping-ill virus infection of grouse; it therefore seems possible that louping-ill virus may have at least been involved in the syndrome termed "Grouse Disease". There is no available data on the present day distribution or abundance of grouse in Scotland. However, it does appear that grouse have virtually disappeared from some areas of the west coast of Scotland where previously they were comparatively abundant. Much of this decline has been attributed to competition from sheep for the available vegetation (Dr. Robert Moss, personal communication). In the light of present findings the association of sheep with the decline of the grouse population suggests that louping-ill virus may also have been a contributory factor.

Louping-ill virus has been shown to be able to pass trans-stadially in a variety of ticks; Ripicephalus appendiculatus (Alexander and Neitz, 1933) and Haemophysalis anatolicum (Swanepoel, 1968) which suggests that ticks other than I. ricinus could transmit virus. Of the 15 species of Ixodes other than I. ricinus which have been recorded in the British Isles at least ten probably occur in Scotland (Arthur, 1963). It is therefore feasible that an occult zoonosis occurs in wild vertebrates and

one or more of these tick species. This can only be suggested tentatively as a possibility but the marked susceptibility of the grouse to infection and the high viraemia that develops in this species does suggest that further studies should be performed with other birds. The potential relevance of this is also suggested from the fact that, of the ten species of Ixodes other than I. ricinus found in Scotland, seven are associated primarily with birds.

Although there is no conclusive evidence as to the role of avian species in the maintenance of members of the tick-borne encephalitides numerous authors have suggested their importance (Federov, 1958; Naumov, Levcovich, Rzhakhova, 1963; Korenberg, 1966; Work, 1962; Erneck, Kožuch, Lichard and Nosek, 1968; Erneck et al, 1971). In addition the viraemias that developed in wild duck (Anas platyrynchos), coots (Fulica atra) and blackbirds (Turdus merula) following peripheral inoculation with tick-borne encephalitis virus suggested that these species could infect ticks (Erneck et al, 1969; Tongeren and Timmers, 1961; Saikku, 1973). These findings support the suggestion that the relationship of louping-ill virus to other bird species should be considered in any subsequent epidemiological investigations.

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Appendix 2:1.

The complete results for testing for neutralising antibody in serum from a sheep experimentally inoculated with Louping-ill virus are presented below. The plaque count observed with the pre-inoculation serum is represented by Po and the observed count of the control mixtures on the day of test by Co. Similarly, the plaque count observed with sera collected on the 3rd, 4th and 5th days after inoculation are represented by P3, P4 and P5 respectively and the respective control mixtures by C3, C4 and C5. The level of natural inhibitor (N) in the pre-inoculation sera is derived from $\frac{Po}{Co}$. The level of specific neutralising activity ($\frac{S}{N}$) is derived from the equation $\frac{S}{N} = \left(\frac{Co}{P} \times \frac{1}{N}\right) - 1$. Specific neutralising activity was first detected at low levels on day 3 and at high levels on day 4 and 5 after infection.

$P_o = 53.0$

$C_o = 76.8$

$N = \frac{53.0}{76.8} = 0.6901$

$P_3 = 38.4$ $\quad \bar{P}_3 = \frac{76.8}{71.8} \times 38.4 = 41.07$

$C_3 = 71.8$ $\quad \frac{S}{N} = \left(\frac{41.07}{76.8} \times \frac{1}{0.6901}\right) - 1 = -0.2251$

$P_4 = 5.0$ $\quad \bar{P}_4 = \frac{76.8}{74.3} \times 5.0 = 5.16$

$C_4 = 74.3$ $\quad \frac{S}{N} = \left(\frac{5.16}{76.8} \times \frac{1}{0.6901}\right) - 1 = -0.9027$

$P_5 = 0.8$ $\quad \bar{P}_5 = \frac{76.8}{70.0} \times 0.8 = 0.88$

$C_5 = 70.0$ $\quad \frac{S}{N} = \left(\frac{0.88}{76.8} \times \frac{1}{0.6901}\right) - 1 = -0.9836$

Appendix 2:1.

The complete results for testing for neutralizing antibody in serum from a sheep experimentally inoculated with louping-ill virus are presented below. The plaque count observed with the pre-inoculation serum is represented by Po and the observed count of the control mixtures on the day of test by Co. Similarly, the plaque count observed with sera collected on the 3rd, 4th and 5th days after inoculation are represented by P3, P4 and P5 respectively and the respective control mixtures by C3, C4 and C5. The level of natural inhibitor (N) in the pre-inoculation sera is derived from $\frac{Po}{Co}$. The level of specific neutralizing activity ($\frac{S}{N}$) is derived from the equation $\frac{S}{N} = \left(\frac{\overline{Py}}{Co} \times \frac{1}{N}\right) - 1$. Specific neutralizing activity was first detected at low levels on day 3 and at high levels on day 4 and 5 after infection.

$Po = 53.0$

$Co = 76.8$

$N = \frac{53.0}{76.8} = 0.6901$

$P_3 = 38.4$ $\qquad \overline{P}_3 = \frac{76.8}{71.8} \times 38.4 = 41.07$

$C_3 = 71.8$ $\qquad \frac{S}{N} = \left(\frac{41.07}{76.8} \times \frac{1}{0.6901}\right) - 1 = -0.2251$

$P_4 = 5.0$ $\qquad \overline{P}_4 = \frac{76.8}{74.3} \times 5.0 = 5.16$

$C_4 = 74.3$ $\qquad \frac{S}{N} = \left(\frac{5.16}{76.8} \times \frac{1}{0.6901}\right) - 1 = -0.9027$

$P_5 = 0.8$ $\qquad \overline{P}_5 = \frac{76.8}{70.0} \times 0.8 = 0.88$

$C_5 = 70.0$ $\qquad \frac{S}{N} = \left(\frac{0.88}{76.8} \times \frac{1}{0.6901}\right) - 1 = -0.9835$

Appendix 2:2. Natural inhibitors in control sheep serum collected on three different occasions.

Sheep No.	N values	S/N values day 3	S/N values day 5
3N05	0.5095	-0.0766	-0.1602
3N15	0.6857	+0.0185	-0.0187
3N19	0.6257	+0.0281	-0.0622
3N23	0.6029	-0.0313	+0.0841
3N36	0.6285	-0.0730	+0.0963
3N40	0.7142	-0.0014	+0.0024
3N42	0.7942	-0.0532	-0.0533
3N46	0.6541	+0.1605	-0.0250
Mean	0.6518	-0.0035	-0.0170
± SE	±0.0293	±0.0250	±0.0289

Appendix 2:3. The S/N values calculated from tests performed on different occasions on the same samples.

Sheep No.	Inoculum	Day p.i.	S/N value Test No.1	Test No.2	Difference
3N05	NMB	3	-0.0766	+0.0185	-0.0951
3N06	SB526	3	-0.0855	-0.1175	+0.0320
3N08	"	3	-0.1758	-0.2356	+0.0598
3N11	"	3	-0.2436	-0.2273	-0.0163
3N12	"	3	-0.2244	-0.1657	-0.0587
3N17	"	3	-0.3640	-0.3305	-0.0335
3N20	"	3	-0.1640	-0.1750	+0.0110
3N24	"	3	-0.0997	-0.1887	+0.0890
3N47	"	3	-0.2251	-0.2200	-0.0051
3N25	"	4	-0.8474	-0.8260	-0.0214
SN06	"	5	-0.9697	-0.9662	-0.0035
3N12	"	5	-0.9288	-0.9203	-0.0085
3N16	"	8	-0.8876	-0.9404	+0.0528
3N20	"	5	-0.7117	-0.6276	-0.0841
3N27	"	5	-0.8337	-0.8330	-0.0007
3N46	NMB	5	-0.0250	-0.0331	+0.0081
3N47	SB526	5	-0.9759	-0.9835	+0.0076

NMB - normal mouse brain

SB/526 - isolate of louping-ill virus

Appendix 3:1. The titres of virus detected in sheep plasmas following inoculation with louping-ill virus expressed as $\log_{10}$ pfu.0.2 ml in susceptible sheep.

Sheep No.	Day post-inoculation 1	2	3	4	5	6	7
3N04	N	N	N	N	N	N	N
3N06	1.32	2.24	4.47	4.76	1.00	N	N
3N07	2.03	4.38	6.12	7.33	4.76	1.31	$\bar{1}$.70
3N08	2.50	3.56	4.98	4.21	$\bar{1}$.70	N	N
3N09	2.36	4.64	4.85	4.60	1.78	0.53	N
3N11	2.08	4.30	5.68	4.18	1.06	0.26	N
3N12	1.95	4.59	6.20	7.00	3.90	0.10	0.51
3N14	3.40	5.65	7.51	7.34	4.72	3.18	D
3N16	2.00	4.11	4.65	5.69	2.08	N	N
3N17	2.11	4.28	5.76	5.79	2.20	0.51	N
3N21	2.15	4.32	6.04	7.11	3.62	1.36	0.78
3N22	3.63	5.68	6.26	4.81	1.99	$\bar{1}$.90	0.08
3N24	2.00	3.91	5.79	6.62	4.36	0.83	N
3N27	1.26	2.94	5.15	6.48	4.80	1.38	$\bar{1}$.60
3N29	1.94	3.85	5.87	4.15	0.78	$\bar{1}$.60	N
3N31	1.77	2.78	4.76	5.89	3.79	0.51	N
3N32	1.67	3.52	5.04	5.18	1.62	$\bar{1}$.90	N
3N34	1.06	4.30	5.08	6.08	1.48	$\bar{1}$.60	N
3N38	2.18	4.26	6.00	6.15	2.45	$\bar{1}$.30	N
3N39	1.85	3.81	5.65	3.81	2.95	0.97	$\bar{1}$.30
3N41	N	1.93	3.15	2.28	0.11	N	N
3N45	2.43	5.59	6.90	5.96	2.42	0.26	$\bar{1}$.60
Mean	2.08	4.03	5.52	5.50	2.46	0.58	$\bar{1}$.88
± SE	±0.14	±0.22	±0.20	±0.29	±0.34	±0.24	±0.22

N = No virus detected.

Appendix 3:2 The titres of virus detected in sheep plasmas following inoculation with louping-ill virus, expressed as $\log_{10}$ pfu. per 0.2 ml in sheep that survived.

Sheep No.	Day post-inoculation 1	2	3	4	5	6	7
3N02	T.52	2.73	3.55	1.43	.70	N	N
3N10	1.97	3.54	5.00	4.00	N	N	N
3N13	1.93	3.84	5.83	4.54	0.30	N	N
3N18	2.04	3.46	4.83	4.51	0.81	N	N
3N20	1.90	3.45	4.86	5.84	0.86	N	N
3N25	1.78	3.89	4.78	3.96	0.30	N	N
3N26	1.24	2.30	3.73	5.56	2.34	N	N
3N33	1.88	3.20	4.72	4.73	0.48	N	N
3N37	2.11	3.30	4.72	4.32	.70	N	N
3N44	1.93	3.62	4.86	3.40	0.95	N	N
3N47	1.18	2.53	3.53	1.60	.70	N	N
Mean	1.59	3.26	4.58	3.99	0.51		
± SE	±0.23	±0.16	±0.21	±0.42	±0.25		
P	>0.10	>0.025	>0.01	>0.01	>0.001		

P = Difference in levels of viraemia in survivors and susceptible animals as calculated by the student t-test.

N = No virus detected.

Appendix 3:3. Reciprocal HI antibody titres to louping-ill virus in sera from susceptible sheep.

Sheep No.	Day post-inoculation											
	*5		6		7		8		9		10	
	PBS	2-ME	PBS	2-ME	PBS	2-ME	PBS	2-ME	PBS	2-ME	PBS	2-ME
3N04	<10	<10	40	<10	80	40	D					
06	<10	<10	80	<10	640	80	>1280	>80	>2560	320	2560	>320
07	<10	<10	40	<10	320	10	D					
08	20	<10	320	<10	2560	>40	5120	>160	D			
09	<10	<10	80	10	640	20	>1280	40	>1280	160	D	
11	<10	<10	80	<10	640	>10	1280	80	2560	80	D	
12	<10	<10	>20	<10	320	>10	D					
14	<10	<10	10	<10	D							
16	<10	<10	40	<10	1280	80	D					
17	<10	<10	>40	<10	640	20	>5120	>20	D			
21	<10	<10	20	<10	160	10	1280	20	>640	>20	D	
22	<10	<10	160	<10	2560	40	D					
24	<10	<10	10	<10	640	20	D					
27	<10	<10	10	<10	320	10	1280	>10	D			
29	<10	<10	80	<10	1280	20	2560	80	D			
31	<10	<10	20	<10	640	20	2560	>40	>1280	160	>1280	320
32	<10	<10	80	<10	>1280	40	5120	>80	D			
34	<10	<10	320	10	1280	40	2560	80	D			
38	<10	<10	160	<10	640	>20	>2560	40	D			
39	<10	<10	10	<10	1280	40	>1280	20	D			
41	<10	<10	>20	<10	160	20	>320	160	D			
45	<10	<10	>40	10	>320	>10	>1280	>80	D			

D = animal dead.

* = Treated with PBS as a control.

Appendix 3:4. Reciprocal HI antibody titres to louping-ill virus in sera from sheep that survived.

Sheep No. *	Day post-inoculation 5		6		7		8		9		10		16		22		28	
	PBS	2-ME	PBS	2-ME	PBS	2-ME	PBS	2-ME	PBS	2-ME	PBS	2-ME	PBS	2-ME	PBS	2-ME	PBS	2-ME
3N02	<10	<10	80	10	640	20	>1280	80	>1280	>80	>640	80	>1280	>1280	>1280	>1280	>1280	>1280
10	40	<10	>640	20	5120	80	10240	640	5120	>320	>2560	>640	>2560	2560	5120	>2560	2560	>1280
13	40	<10	>640	>10	>5120	40	10240	160	>5120	160	2560	>160	>2560	>640	>640	>320	>1280	1280
18	<10	<10	160	10	2560	80	1280	80	>1280	>80	>1280	>160	640	640	320	>160	>320	>320
20	40	10	>160	10	2560	80	2560	640	>2560	320	>1280	320	5120	>1280	2560	>2560	>1280	>1280
25	20	10	>320	10	2560	20	2560	>40	>1280	>40	1280	80	>640	>320	>160	160	320	>160
26	<10	<10	>40	<10	1280	20	1280	80	1280	160	1280	160	>640	>640	>640	>640	>640	640
33	20	10	>160	20	2560	80	2560	>160	>1280	160	1280	>160	1280	320	1280	640	640	640
37	<10	<10	160	<10	1280	>20	2560	> 40	2560	>80	1280	80	1280	320	>320	160	640	640
44	10	10	>40	10	>640	>20	2560	160	>1280	>160	1280	>160	2560	2560	5120	>1280	1280	1280
47	40	<10	>160	10	640	40	1280	80	640	80	>320	80	640	>320	>320	>160	>320	>320

* Treated with PBS as a control.

Appendix 3:5. Neutralizing activity to louping-ill virus in sera of sheep that succumbed.

Sheep No.	N. Values	$\frac{S}{N}$ values Day 3	Day 4	Day 5
3N06	1.0090	-0.0855	-0.5456	-0.9697
07	0.7030	+0.0358	0:1302	-0.9170
08	0.6864	-0.1758	-0.8102	-0.9718
09	0.7332	-0.2532	-0.2242	-0.9298
11	1.0500	-0.2273	-0.7283	-0.9537
12	0.8593	-0.1657	-0.2623	-0.9288
14	0.7227	-0.0325	-0.2146	-0.8808
16	0.7174	-0.0004	-0.5529	-0.8876
17	0.7411	-0.0122	-0.3305	-0.7950
21	0.7285	-0.01466	+0.0318	-0.8816
22	0.8068	-0.2181	-0.7237	-0.9414
24	0.6438	-0.0997	-0.0542	-0.8401
27	0.9040	-0.0727	NT	-0.8330
29	0.6307	+0.0067	-0.6971	-0.9702
31	0.7174	+0.0287	-0.3259	-0.7943
32	0.9145	-0.2113	-0.5863	-0.9391
34	0.5229	-0.0415	-0.8148	-0.9641
39	0.6702	-0.0338	-0.3254	-0.8858
41	0.7103	-0.1373	NT	-0.8904
45	0.7660	+0.0194	-0.2938	-0.9055
Mean	0.7618	-0.0845	-0.4215	-0.9030
$\pm$ SE	$\pm$0.0287	$\pm$ 0.0210	$\pm$ 0.0626	$\pm$ 0.0122

Appendix 3:6. Neutralizing activity to louping-ill virus in sera of sheep that survived.

Sheep No.	N. Values	$\frac{S}{N}$ Values Day 3	Day 4	Day 5
3N02	0.5335	-0.0540	-0.6760	-0.9061
10	0.9660	-0.3834	-0.8642	-0.9778
13	0.6754	-0.1612	-0.8517	-0.9795
18	0.4914	+0.0851	-0.6122	-0.9152
20	0.7332	-0.1640	-0.7117	-0.9621
25	0.8669	-0.2477	-0.8474	-0.9521
26	0.7542	-0.0720	-0.4220	-0.9189
33	0.4872	+0.0215	-0.6093	-0.9315
37	0.7306	-0.2452	-0.7029	-0.8858
44	0.6622	+0.1208	-0.8335	-0.9538
47	0.6901	-0.2251	-0.9027	-0.9835
Mean	0.6903	-0.1204	-0.7302	-0.9423
± SE	±0.0445	±0.0468	±0.0440	±0.0095
P	> 0.2	> 0.4	< 0.005	< 0.05

P = Difference between survivors and susceptibles as calculated by the student t-test.

Appendix 3:7. Reciprocal HI antibody titres in sera collected from sheep that had been inoculated once with louping-ill virus 3 to 5 years previously.

Sheep No.	Months after inoculation	Titre
1C32	64	80
1C14	53	160
5C20	51	640
5C21	51	160
2F81	44	320
2591	44	320

Appendix 3:8. Concentrations of IgG in sera of sheep derived by the single ring diffusion technique.

Sheep No.	Ring Diameter	Log_{10} concentration of protein at 1/200	mg IgG/100ml
3N22	5.10	0.72+	1060
3N08	4.40	0.58	950
3N32	5.51	0.80	1260
3N34	4.10	0.52	660
3N21	NE	NE	NE
3N11	5.15	0.73	1070
3N31	6.11	0.92	1660
3N02	5.31	0.76	1150
3N20	6.01	0.90	1600
3N44	5.76	0.85	1420
3N18	5.91	0.88	1500
3N47	5.56	0.81	1300
3N33	5.51	0.80	1260
3N03	4.40	0.58	950
3N23	4.40	0.58	950
3N42	6.66	1.03	2160
3N43	4.95	0.69	980
3N46	5.05	0.71	1020

+ derived from the equation $Y = 0.1990X - 0.2955$ where X = ring diameter and Y is log_{10} concentration of protein.

NE = not examined.

Appendix 3:9. Concentrations of IgG in sheep CSF derived by the single ring diffusion technique.

Sheep No.	Ring Diameter	Log_{10} concentration of protein	mg IgG/100ml
3N22	5.96	0.89[+]	7.7
08	4.20	0.54	3.5
32	4.40	0.58	3.8
34	6.06	0.91	8.2
21	5.00	0.70	5.0
11	4.30	0.56	3.6
31	4.67	0.64	4.4
02	6.11	0.92	8.3
20	7.16	1.13	13.5
44	6.31	0.96	9.2
18	4.65	0.63	4.3
47	6.16	0.83	6.7
33	4.85	0.67	4.7
03	3.19	0,34	2.17
23	4.40	0.58	3.8
42	4.30	0.56	3.6
43	4.20	0.54	3.5
46	3.75	0.45	2.8

[+] derived from the equation $Y = 0.1990X - 0.2955$ where X = ring diameter and Y is log_{10} concentration of protein.

Appendix 3:10. Concentrations of IgM in sera from sheep derived by the single ring diffusion technique.

Sheep No.	Ring Diameter	Log_{10} concentration of protein at 1/20	mg IgM/100ml
3N22	4.39	0.74+	109
3N08	4.19	0.65	89
3N32	4.30	0.70	101
3N34	NE	NE	NE
3N21	4.39	0.74	109
3N11	4.26	0.68	96
3N31	4.62	0.84	137
3N02	4.06	0.59	77
3N20	4.48	0.78	120
3N44	4.55	0.81	130
3N18	4.35	0.72	105
3N47	4.25	0.67	94
3N33	4.48	0.78	121
3N03	4.37	0.73	108
3N23	4.45	0.72	105
3N42	4.55	0.81	128
3N43	NE	NE	NE
3N46	4.71	0.88	153

+ derived from the equation $Y = 0.4444X - 1.2123$ where X = ring diameter and Y is log_{10} concentration of protein.

NE= not examined.

Appendix 3:11. Concentrations of IgM in sheep CSF derived by the single ring diffusion technique.

Sheep No.	Ring diameter	Log_{10} concentration of protein	mg of IgM/100 ml
3N22	5.73	0.71+	5.1
08	4.01	0.57	3.7
32	4.25	0.67	4.7
34	4.25	0.67	4.7
21	3.99	0.56	3.6
11	4.19	0.65	4.5
31	3.92	0.53	3.4
02	-		
20	-		
44	-		
18	-		
47	-		
33	-		
03	-		
23	-		
42	-		
43	-		
46	-		

+ derived from the equation $Y = 0.4444X - 1.2123$ where X = ring diameter and Y is log_{10} concentration of protein.

- = No precipitin ring detected.

Appendix 4:1. The HI antibody response in lambs that received colostral antibody and were challenged with louping-ill virus.

Group No.	Sheep No.	Day post-inoculation -1	+2	+4	+6	+8	+10	+14	+21	+42	+71	+139	+186
1	C/191	20+	20	10	10	10	10	10	10	10	T	T	10
HI antibody	155	20	10	10	>10	20	160	80	40	>80	>40	>40	40
present	156	20	20	>10	20	40	160	320	>80	40	40	40	>20
when	192	20	10	10	10	20	40	80	>20	20	10	T	10
challenged.	195	>20	20	10	10	10	10	10	<10	<10	<10	<10	<10
2	C/147	<10	<10	<10	<10	80	>80	640	80	320			
Found negative	149	<10	<10	<10	10	80	>80	160	320	320			
for HI antibody	151	<10	<10	<10	<10	160	>640	>2560	2560	>1280			
20 days before	199	<10	<10	<10	10	>160	>160	160	80	>80			
challenge.	201	<10	<10	<10	<10	>160	320	1280	1280	5120			
3	C/143	<10	<10	<10	20	>2560	2560	5120	640	640			
Found negative	157	<10	<10	<10	20	2560	2560	>2560	>320	320			
for HI antibody	158	<10	<10	<10	<10	640	2560	D					
48 days before	206	<10	<10	<10	80	160	160	640	320	>320			
challenge.	210	<10	<10	<10	80	1280	320	2560	640	>640			
4	C/185	<10	<10	<10	40	>2560	2560	>2560	320	640			
Found negative	190	<10	<10	<10	320	>2560	>2560	5120	>2560	2560			
for HI antibody	193	<10	<10	<10	>80	2560	2560	>2560	1280	1280			
76 days before	213	<10	<10	<10	10	2560	D						
challenge	214	<10	<10	<10	<10	>320	640	1280	>640	640			
5	C/196	<10	<10	<10	80	>1280	1280	1280	320	>320	320	160	320
No colostral	197	<10	<10	<10	>40	>2560	1280	1280	>1280	>1280	>1280	>320	640
HI antibody	198	<10	<10	<10	160	>1280	640	>640	640	>640	640	160	320
received	226	<10	<10	<10	160	2560	>2560	10240	2560	1280	1280	>640	640
	291	<10	<10	<10	10	2560	>2560	10240	>2560	2560	1280	80	160

+ Reciprocal HI antibody titre. T = Incomplete inhibition detected at a dilution of 1/10.
D = Lamb dead.

Appendix 5:1. The titres of virus detected in the blood of red grouse (Lagopus scoticus) following inoculation with louping-ill virus.

rouse	Day post - inoculation							
No.	1	2	3	4	5	6	7	8
T044	2.60+	3.58	4.45	5.59	3.90	D		
T055	2.71	2.70	5.30	5.69	4.94	4.22	3.53	N
T094	2.98	4.04	5.61	5.15	5.18	3.87	4.72	D
T096	2.62	2.60	3.85	4.59	3.74	3.74	D	
T126	2.15	4.15	4.40	6.03	6.43	D		
T133	2.85	4.87	5.46	6.88	6.77	D		
T140	2.74	3.51	5.23	4.65	5.04	4.64	4.45	4.11
T144	2.92	2.98	5.00	4.30	D			
T147	3.19	4.13	5.46	4.93	5.05	4.66	D	
T148	2.54	3.99	5.74	4.85	4.54	D		
T158	2.95	3.74	5.49	4.90	4.81	4.74	4.15	2.74
T160	2.23	3.60	5.33	D				
T162	2.23	4.11	5.06	5.80	6.06	5.29	N	N
T206	3.28	4.95	4.70	5.00	4.17	D		
T208	3.37	4.34	5.31	D				
T226	3.23	3.82	4.85	4.81	5.42	4.41	2.40	NE
T231	2.72	3.70	D					
T232	2.67	3.45	3.77	4.74	4.81	4.70	3.81	0.70
T236	3.31	5.06	5.58	6.00	D			
T237	3.03	3.97	4.70	5.69	5.66	D		
T238	3.46	4.74	4.81	5.45	5.26	4.16	3.15	N
ean	2.85	3.91	5.01	5.28	5.11	4.44	3.75	2.52
SE	0.09	0.15	0.13	0.15	0.21	0.15	0.30	0.99
T051	2.72	2.92	3.87	3.98	3.65	2.22	N	N
T061	2.88	3.35	4.85	4.36	3.81	4.20	2.85	2.18
T083	2.57	2.95	4.18	4.53	3.40	2.84	3.88	N
T228	2.95	3.79	4.34	5.56	4.25	3.08	2.65	N
T234	3.20	3.96	5.04	4.70	4.10	3.30	N	N
T243	2.92	3.71	3.83	4.01	3.26	3.15	2.18	N
ean	2.87	3.45	4.35	4.52	3.75	3.13	2.89	
SE	0.09	0.18	0.20	0.24	0.16	0.26	0.36	
	>0.90	>0.20	>0.02	>0.02	>0.005	>0.001	>0.20	

= bird dead. N = no virus detected. + Log_{10} pfu. per 0.2 ml.

= difference between survivors and susceptibles as calculated by the student t-test.

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PUBLICATIONS

The studies of experimental louping-ill in susceptible sheep were performed in collaboration with Dr. P.C. Doherty. A strict division of responsibility was, however, maintained.
Dr. P.C. Doherty was exclusively responsible for the histological, fluorescent antibody and electron-microscopical investigations. Examination for viral infectivity, antibody assay and immunoglobulin determination reported in this thesis and in the papers incorporated into this section were conducted entirely by the candidate.

EXPERIMENTAL LOUPING-ILL IN SHEEP AND LAMBS

I. Viraemia and the Antibody Response

By

H. W. Reid and P. C. Doherty

Moredun Research Institute, Gilmerton, Edinburgh

INTRODUCTION

Depending on their age and the route of inoculation with louping-ill virus Syrian hamsters showed marked variations in susceptibility, intensity of viraemia and severity of neuropathological changes (Doherty, 1969a). The relationship between these variables, and the additional factor of the serum antibody response, have now been investigated in sheep. The course of viraemia and haemagglutination inhibition (H.I.) antibody production are described in the present communication. The neuropathological studies are reported in a subsequent paper.

MATERIALS AND METHODS

Animals. The experimental animals comprised twenty 9-month-old Scottish Blackface (B.F.) and B.F. X sheep, which had been purchased, and twelve 3-day-old Cheviot and B. F. cross lambs which were bred on the Institute farms. All animals had been born and reared in tick-free environments and were without serum H.I. antibodies to louping-ill virus. The lambs were allowed to suckle for the first 3 days of life and were then maintained on a milk substitute diet*, in pens fitted with infra-red heater lamps.

Inoculation. Two isolates of louping-ill virus (SB526 and SB527) were recovered by inoculating brain suspensions from clinically affected sheep intracerebrally (i.c.) into mice. These first passage mouse brains were homogenised as a 10 per cent. w/v suspension in 10 per cent. inactivated horse serum saline (H.S.S.) and stored in aliquots of 0·25 ml. at −70°C. A similar control preparation was made from normal mouse brains. Clarified inocula were prepared by diluting the thawed suspensions 1 :100 in H.S.S. and centrifuging at 1,200 *g* for 15 minutes at +4°C. Groups of 4 sheep were inoculated i.c. with 1·0 ml. into the right cerebral hemisphere or subcutaneously (s.c.) with 10·0 ml. into the medial aspect of the right thigh. Thus 8 sheep were injected with each virus inoculum. Groups of 4 lambs were injected i.c. (0·5 ml.) or s.c. (5·0 ml.) with the SB526 isolate. Two sheep and 2 lambs were inoculated i.c. with control material and the same number were inoculated s.c. Each virus inoculum was titrated i.c. in mice (Doherty, 1969b). Sheep inoculated i.c. with SB526 and SB527 received $1{\cdot}9 \times 10^6$ and $9{\cdot}5 \times 10^5$ mouse infective doses respectively, while lambs were given $2{\cdot}5 \times 10^6$ infective doses of SB526. Inocula administered s.c. contained 10 times these amounts. Animals developing severe neurological symptoms were killed in extremis and the survivors were killed at 20 days after inoculation.

Viraemia. One ml. samples of jugular blood were withdrawn 6 hours after inoculation, and subsequently at 12 hourly intervals for 10 days. Each sample was immediately diluted 1 :5 in chilled H.S.S. before clotting occurred, and two 2·5 ml.

* 'Nutrilamb', Scottish Agricultural Industries Ltd., Edinburgh.

aliquots were stored at −70°C. until all specimens had been collected. One aliquot from each pair was thawed, centrifuged at 1,200 *g* and the supernate tested for presence of virus by i.c. inoculation into 5 random bred 3 week old white mice. The duplicates of samples proved positive were thawed and ten-fold dilutions made in H.S.S., to 10^{-4} of original blood for specimens from sheep or 10^{-6} for specimens from lambs, and titrated in mice (Doherty, 1969b).

Virus titres were calculated by the method of Reed and Muench (1938) and expressed as the reciprocal of the $\log_{10}$ ID_{50} per 0·03 ml. of original blood. If virus was detected at a level that was too low to express as a titre it was said to be present in trace (T) amounts.

Serology. Blood samples (8·0 ml.) were collected immediately prior to inoculation and at 5, 10, 15 and 20 days thereafter. In addition, terminal samples were taken from all moribund animals. Blood was allowed to clot at room temperature and kept overnight at +4°C. Serum was pipetted off, centrifuged at 1,000 *g* for 15 minutes at room temperature and the supernate stored at −70°C.

Haemagglutination inhibition serum titres were determined, following kaolin extraction and gander (♂ *Anseris anseris*) red blood cell absorption in standard leucite W.H.O. plates. Four haemagglutinating units of a mouse brain antigen were used and 50 per cent. end points were assessed by the method of Clarke and Casals (1958). After an initial test all positive sera were treated in each of two ways, to assess relative activity in the I_gM and I_gG antibody classes.

(i) Each serum sample was treated with 2-mercaptoethanol (2ME)† by adding 0·05 ml. of a 2 molar solution of 2ME to 0·45 ml. of serum which was kept for 20 hours at 4°C. A further 0·45 ml. of serum was treated with P.B.S. as a control.

(ii) A specimen of each serum was kept at 64·5°C. for 30 minutes in a sealed container, while another specimen was kept at room temperature.

These treatments are similar to those used by Jonas (1969). The samples were then extracted, absorbed and tested for H.I. activity as described above.

Microimmunoelectrophoresis. Pooled sera were subjected to electrophoresis (Scheidegger, 1955) both before and after exposure to heat or 2ME, in order to confirm inactivation of I_gM class antibody by these treatments. The origin was charged with 5μL of serum and electrophoresis was maintained for 100 min., with a potential difference of 150V. and a current of 3 to 4 m.a. per slide.

The troughs were charged with 0·1 ml. of rabbit anti-whole sheep serum and allowed to react for 48 hours. The slides were then soaked in isotonic saline for 48 hours, dried, stained with 0·1 per cent. azocarmine B and fixed with 5 per cent. acetic acid. Precipitin lines were identified using the nomenclature recommended by the W.H.O. Committee on Immunoglobulins (1964).

RESULTS

Clinical

All of the animals inoculated i.c. with virus developed severe neurological symptoms. Typically there was a progression of symptoms from slight ataxia to complete flaccid paralysis over a 6 to 18 hour interval. Animals were killed in extremis at from 92 to 153 hours after inoculation. The times for individuals are given in Table 1. The mean values for lambs inoculated i.c. with SB526 were significantly less ($P<02$) than those from sheep similarly treated. There was no difference ($P<50$) between sheep given SB526 or SB527 i.c.

As shown in Table 2 one sheep (3K45) and 1 lamb (106) that had been

† Koch-Light Laboratories Ltd., Colnbrook, Bucks., England.

TABLE 1

VIRAEMIA* IN ANIMALS INOCULATED I.C.

Virus isolate		*SB527*				*SB526*				*SB526*			
Animals		*Sheep*				*Sheep*				*Lambs*			
		3K30	*3K36*	*3K38*	*3K35*	*3K34*	*3K32*	*3K37*	*3K33*	*109*	*110*	*642*	*107*
	6	0	0	0	0	0	0	T	0	0	0	0	0
	18	0	0	T	0	0	0	0	0	0	0	0	0
Hours	30	0	0	0	0	T	T	T	0	0	0	0	T
	42	0	0	0	0	T	1·5	1·5	T	T	T	T	T
after	54	0	T	T	0	0·5	2·0	2·4	T	1·4	T	T	2·6
	66	T	T	T	0	0·6	3·0	3·6	T	1·2	T	1·4	3·8
inoculation	78	T	1·4	2·0	0	1·8	1·5	>4·0	1·2	2·8	2·8	2·4	5·2
	90	0	2·4	2·4	0	1·0	1·6	>4·0	2·3	2·8	3·0	2·2	4·6
	102	0	2·4	T	0	T	1·2	>4·0	1·5	(92)	(94)	(97)	3·7
	114	0	T	0	0	0	0	3·0	1·7				2·2
	126	(117)	0	0	0	(117)	0	1·8	T				(116)
	138		(121)	(128)	0		(127)	T	0				
	150				(139)			0	0				
								(152)	(153)				

* Expressed as $\log_{10}$ ID_{50}/0·03 ml. of original blood.

T = Trace 0 = No virus isolated ( ) = Time at which moribund animals were killed.

TABLE 2

VIRAEMIA* IN ANIMALS INOCULATED S.C.

Virus isolate		*SB527*				*SB526*				*SB526*			
		Sheep				*Sheep*				*Lambs*			
Animals		*3K43*	*3K44*	*3K42*	*3K39*	*3K45*†	*3K41*	*3K29*	*3K28*	*106*†	*641*	*108*	*104*
	6	0	0	0	0	0	0	0	0	0	0	0	0
	18	0	0	0	0	T	0	0	T	T	T	T	0
Hours	30	T	0	0	0	1·6	0	T	T	T	T	0	T
	42	T	0	T	0	1·6	0	0·6	T	0	1·7	T	T
after	54	T	T	0·6	0	2·0	T	1·5	T	2·5	2·3	T	T
	66	1·5	T	T	T	2·7	T	T	1·8	2·7	0·8	T	2·6
inoculation	78	2·2	T	2·5	T	2·0	T	1·8	2·4	3·3	T	1·5	1·5
	90	1·5	T	1·6	T	1·5	T	0·8	1·5	2·6	T	2·3	1·7
	102	T	T	2·2	0	T	T	0·6	T	2·2	0	1·5	T
	114	T	0	1·6	0	0	T	T	T	T	T	2·4	T
	126	T	T	T	0	0	0	0	0	T	0	1·2	0
	138	0	0	T	0	0	0	0	0	T	0	2·5	0
	150	0	0	T	0	0	0	0	0	T	0	T	0
	162	0	0	T	0	0	0	0	0	0	0	T	0
	174	0	0	0	0	0	0	0	0	0	0	0	0

* Expressed as $\log_{10}$ ID_{50}/0·03 ml. of original blood.

T = Trace 0 = No virus isolated

† Sheep 3K45 and lamb 106 were killed in extremis at 171 and 217 hours after inoculation respectively. The other animals survived.

inoculated s.c. with SB526 were killed, when moribund, at 171 and 217 hours respectively. The other 10 that had been given virus s.c. survived until the experiments were terminated on day 20. However non-fatal neurological symptoms were seen in 4 of these animals, comprising 2 sheep and 1 lamb inoculated with SB526 and 1 sheep inoculated with SB527. Sheep 3K41 developed a permanent non-progressive posterior paresis from day 12 and 3K28 was slightly ataxic from days 8 to 10 as was 3K43 on days 11 and 12. Lamb 641 was ataxic from days 10 to 15.

Viraemia

The duration and magnitude of viraemia in the 24 sheep and lambs inoculated with virus is shown in Tables 1 and 2. Concentrations of at least 1,000 mouse infectious doses per ml. of blood were recovered, on at least one occasion, from 19 of these animals. Circulating virus was detected in smaller amounts in 4 others, and was not found at all in 1 sheep that was given SB527 i.c. or any of the 8 controls.

The degree of viraemia tended to be greater in sheep that were inoculated i.c. with SB526 than in those that were given SB527 i.c., though all animals succumbed and incubation times were similar in both groups (Table 1). Viraemia terminated before the onset of severe symptoms in all of the 8 sheep that were inoculated i.c., whereas it was still detected at death in the 4 lambs that were inoculated i.c. Although there were marked individual variations in duration and magnitude of viraemia in animals that were given virus s.c. there was no obvious difference between the 3 experimental groups (Table 2). The maximum titres of virus recovered from the 2 that succumbed were marginally higher than those from the 10 survivors, but this could not be considered significant.

Antibody Response

Serum H.I. antibodies were demonstrated in all animals inoculated s.c. with virus, but were not found in any of those that were inoculated i.c. or in the controls. On immunoelectrophoresis the I_gG precipitin line was found to be unaffected by treatment with either heat or 2ME, but the I_gM precipitin line was invariably eliminated. The two treatments gave similar results (Table 3). Thus H.I. titres of treated samples may be reasonably regarded as representing activity in the I_gG class of antibody alone, whereas the differences between treated and untreated samples may be considered as due to I_gM.

The predominant H.I. activity in sera collected early in the course of infection was due to I_gM. Such antibody was detected on day 5 in 3 of 4 sheep inoculated s.c. with SB526, but was not found in any of the 4 lambs. All animals were serologically positive by day 10, when the total level of activity was at a maximum in 5 of 7 surviving sheep, but not in any of 3 surviving lambs. Maximum I_gG activity, as assessed by 2ME treatment, was first recorded on day 15 (6 sheep and 1 lamb) or day 20 (1 sheep and 2 lambs).

DISCUSSION

The virus circulated in all 12 animals that were inoculated s.c. Similar results were recorded by Swanepoel (1968) and Zlotnik, Keppie and Grant (1970) from

TABLE 3

THE H.I. ANTIBODY RESPONSE IN ANIMALS INOCULATED S.C.

Virus isolate		*SB527*				*SB526*				*SB526*			
Animals		*Sheep*				*Sheep*				*Lambs*			
		3K43	*3K44*	*3K42*	*3K39*	*3K45*	*3K41*	*3K29*	*3K28*	*106*	*641*	*108*	*104*
Untreated	5†	0	0	0	20	80	20	10	0	0	0	0	0
	10	2560	640	5120	1280	2560	640	1280	2560	640	320	640	640
	15	5120	320	5120	1280	(7)	1280	1280	2560	(9)	640	2560	2560
	20	2560	640	2560	640		2560	640	2560		320	320	2560
Heat	5	0	0	0	0	0	0	0	0	0	0	0	0
	10	20	40	80	40	20	20	160	ND	80	10	20	0
	15	2560	80	2560	160		320	40	ND		80	10	40
	20	2560	160	1280	160		640	80	320		80	40	320
2ME	5	0	0	0	10	0	0	0	0	0	0	0	0
	10	160	20	80	20	80	10	40	80	80	10	20	10
	15	1280	160	2560	80		160	160	640		40	160	160
	20	1280	160	1280	80		1280	80	320		80	80	640

Titres are expressed as reciprocals.

† Days after inoculation.

( ) = Day of terminal sample from moribund animal.

ND = Not determined, as insufficient serum available.

sheep that had been given other isolates of louping-ill virus by the same route. Dissemination by the blood is, as with other arbovirus encephalitides (Johnson and Mims, 1968), probably an essential pre-requisite for infection of nervous tissue and the subsequent development of symptoms of louping-ill.

The SB526 virus was the more virulent of the 2 isolates. In sheep that were inoculated s.c. neurological symptoms were seen in 3 of 4 that were injected with SB526, whereas slight symptoms were observed in only 1 of 4 given SB527. This clinical pattern was not obviously related either to the degree of viraemia or the antibody response in the individuals concerned. However, in sheep that were inoculated i.c. viraemia resulting from exposure to SB526 was much more marked than that following injection of SB527. The degree of viraemia following i.c. inoculation may in some way reflect the inherent virulence of the virus.

Serum H.I. antibody was detected on day 5 in 4 of 8 sheep that had been inoculated s.c., but was not found in any of 8 sheep killed at 5 to 7 days after i.c. exposure. It may be that administration of louping-ill virus by the i.c. route gives a less rapid stimulus to antibody forming mechanisms than s.c. inoculation. Virus given s.c. probably passes by the lymphatics to the regional lymph nodes, where it replicates (Malkova, 1968). It is likely that much of an i.c. inoculum passes directly into the blood (Mims, 1960) and viral antigen may reach antibody forming cells in smaller amounts by less direct routes. An alternative, but less feasible, explanation is that the dose given is extremely critical, for 10 times as many virus particles were injected by the s.c. as by the i.c. route.

In moribund sheep that had been inoculated i.c. serum antibody may have been present at levels that were below the limit of sensitivity of the H. I. test, perhaps because all available antibody was combined with circulating virus. This may explain why virus was not detected in terminal blood samples from these sheep. However, the lambs that were inoculated i.c. were still viraemic at death. The disease was significantly more acute in the younger animals, so there would have been less time for antibody to develop.

Lambs inoculated s.c. developed antibody more slowly than sheep. Comparatively lower immune responses in young animals have also been observed in lambs exposed to other antigenic stimuli (Silverstein, Uhr, Kraner and Lukes, 1963) and in calves inoculated with another group B arbovirus (Sanderson, 1968b). Even so the 3 day old lambs were no more susceptible to s.c. inoculation than adult sheep, and levels of viraemia in the 2 age groups were not obviously different. In this respect sheep differ from the Syrian hamster. The highly susceptible infant-hamster probably has no immune response, for viraemia persists until death on day 8, whereas in the resistant adult viraemia terminates at about 6 days after inoculation (Doherty, 1969a).

In both sheep and lambs the initial serological response was due to I_gM class antibody whereas I_gG class antibody appeared later. A similar pattern was observed in calves that were inoculated with other arboviruses by Sanderson (1968a and b). This sequence has also been observed in sheep injected with other antigens (Merriman and Rice, 1969; Jonas, 1969).

SUMMARY

Quantitative studies of viraemia and the antibody response to louping-ill virus were made in sheep and lambs that had been inoculated intracerebrally or subcutaneously. Viraemia was demonstrated in 23 of the 24 experimental animals and was detected at from 1 to 7 days after inoculation. There was no obvious relationship between susceptibility and degree of viraemia following subcutaneous exposure. Serum haemagglutination inhibiting antibody was first detected at between 5 and 10 days after subcutaneous inoculation, but was not found in any of 8 sheep that were moribund 5 to 7 days after intracerebral injection. The initial activity was associated with I_gM class antibody, whereas I_gG class antibody appeared later. Young lambs developed antibody more slowly than adult sheep, but were no more susceptible to subcutaneous inoculation with virus.

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LOUPING-ILL ENCEPHALOMYELITIS IN THE SHEEP

I. The Relationship of Viraemia and the Antibody Response to Susceptibility

By

H. W. Reid* and P. C. Doherty

Moredun Research Institute, Gilmerton, Edinburgh

INTRODUCTION

Smith, McMahon, O'Reilly, Wilson and Robertson (1964) suggested that the incidence of neurological symptoms in sheep exposed to louping-ill virus depends on the magnitude and duration of viraemia, which is in turn influenced by the rate of development of circulating antibody. Our previous study of louping-ill in the sheep (Reid and Doherty, 1971), which was designed to assess the effects of the route of inoculation and the age of the host on the disease process, gave tentative support for this hypothesis. However only small numbers of animals were used, antibody levels were determined at 5-day intervals and viraemia was measured by inoculating whole blood into mice. We have now developed a sensitive in vitro plaque assay for louping-ill virus, which has made feasible an intensive investigation of the viraemia and antibody responses in a large number of animals.

MATERIALS AND METHODS

Experimental. Six-month-old Scottish Blackface sheep, which were without serum haemagglutination inhibiting (H.I.) antibodies to louping-ill virus, were purchased from a tick-free environment. Using techniques described previously (Reid and Doherty, 1971), 33 were each inoculated subcutaneously (s.c.) with $3{\cdot}0 \times 10^7$ p.f.u. of the SB526 isolate of louping-ill virus and another 8 were given control material. The virus inoculum was prepared from the brains of infant mice injected intracerebrally with blood from viraemic lambs (Reid and Doherty, 1971). Sheep were bled every 24 hours for heparinized† (4·0 i.u. per ml.) whole blood samples and serum samples. A total of 30·0 ml. of blood was collected each day from each sheep. Following separation, tissue cultures were immediately inoculated with 0·2 ml. volumes of plasma. The serum and the remaining plasma were stored at −70°C.

Tissue culture media. Medium A consisted of Hank's balanced salt solution (B.S.S.) containing 100 i.u. per ml. of penicillin and streptomycin‡, 0·02 mg. per ml. of glucose, 0·5 per cent. w/v lactalbumin hydrolysate§, 0·01 per cent. w/v yeast extract§ and 10 per cent. inactivated calf serum. In medium B, Hank's B.S.S. was replaced by Earle's B.S.S. and 0·001 mg. per ml. of folic acid was added. The overlay medium was similar to medium B, but contained only 5 per cent. serum, double strengths of antibiotics and sodium bicarbonate, 100 i.u. per ml. of Mycostatin¶ and 1·5 per cent. w/v of sodium carboxy methyl cellulose.**

* Present address: E.A.V.R.O., Muguga, P.O. Kabete, Kenya.
† "Pullarin", Evans Medical Ltd., Liverpool, England.
‡ B.D.H., Poole, England.
§ Difco Laboratories Ltd., Detroit, Michigan, U.S.A.
¶ E. R. Squibb and Sons, Liverpool, England.
** Koch-Light Laboratories Ltd., Colnbrook, England.

Preparation of monolayers. Cultures of the 1B/RS2 clone 60 pig kidney cell line* were grown in medium A in Roux flasks. The cells were at the 180th to 190th passage and were sub-cultured at 4- or 5-day intervals. A total of $2{\cdot}4 \times 10^6$ cells, in 8·0 ml. of medium B, were dispensed into each 5 cm. disposable plastic petri plate†. Monolayers were formed by incubating at 35°C. for 24 hours, in an atmosphere containing 5 per cent. CO_2.

Plaque assay. The growth medium was removed and 0·2 ml. of fresh undiluted plasma was added to each of 4 or 5 plates. This inoculum was absorbed for 2 hours at 35°C. with frequent rocking. Then, without washing, 6·0 ml. of overlay medium was added to each plate. The cultures were incubated for a further 72 hours, washed in phosphate buffered saline (pH 7·2) and stained with 1·0 per cent. crystal violet‡ in 20 per cent. ethyl alcohol. If a confluent cytopathic effect developed a duplicate sample of frozen plasma was thawed, diluted in medium B and inoculated onto more plates. Plaques were counted and the mean number of p.f.u. per 0·2 ml. of plasma was calculated.

Plaque neutralization test. The Moredun sheep strain of louping-ill virus (LI31) was diluted in medium B, so as to give a count of approximately 70 p.f.u. in control cultures. Sera, heated to 56°C. for 15 mins., were then cooled and 1 vol. of medium B and 1 vol. of virus were added to each sample. The serum-virus mixtures and the control, which consisted of 2 vol. of medium B and 1 vol. of virus, were incubated at 37°C. for 30 mins. These mixtures were then kept on ice until inoculated onto 5 plates, which were processed as described above except that each monolayer was washed with 5·0 ml. of medium B before the overlay was added.

The level of natural inhibitors (N) present in serum was expressed as the fraction of virus remaining following incubation with pre-inoculation serum. Hence $N = \frac{Po}{Co}$, where Po is the observed plaque count following incubation with pre-inoculation serum and Co is the observed plaque count following incubation with medium B on the day of test.

To determine the level of specific neutralizing activity in a serum sample from day y, the observed plaque count following incubation with that serum (Py) was corrected for natural inhibitors present in serum from the individual concerned. Before this could be done it was necessary to adjust Py according to the control plaque count (Cy), for the day when Py was determined, to correspond to the control plaque count (Co) on the day when the pre-inoculation serum was tested. This may be expressed as follows: the adjusted plaque count $(\overline{Py}) = \frac{Co}{Cy} \times Py$. The adjusted fraction of virus following incubation with a serum from day y is therefore $\frac{\overline{Py}}{Co}$. This fraction is due to both specific neutralizing capacity (S) and activity of natural inhibitors (N), hence $\frac{\overline{Py}}{Co} = S + N$.

The specific neutralizing capacity of the serum is expressed by $\frac{S}{N}$. Thus to calculate $\frac{S}{N}$:

$$\frac{\overline{Py}}{Co} \times \frac{1}{N} = \frac{S+N}{N}$$

$$= \frac{S}{N} + 1$$

$$\frac{S}{N} = \left(\frac{\overline{Py}}{Co} \times \frac{1}{N}\right) - 1$$

* Obtained from the Animal Virus Research Institute, Pirbright, Surrey, England.
† Nunc, Algade S, Rosskilde, Denmark.
‡ George T. Gurr Ltd., London, England.

Haemagglutination inhibition test. Both the H.I. test and the treatment of serum with 2-Mercaptoethanol (2ME), which inactivates IgM but not IgG, have been described previously (Reid and Doherty, 1971).

RESULTS

Clinical

Four of the 33 sheep that were inoculated s.c. with virus died and 18 were killed with severe neurological symptoms. These 22 animals, which are referred to as "susceptible", were moribund at from 6 to 11 days (average 8 days) after inoculation. Generally the first symptom observed was slight ataxia, which rapidly progressed to complete flaccid paralysis within a 3- to 5-hour interval. The other 11 survived without symptoms of encephalomyelitis although 2 animals (24 and 25) were chronically debilitated. The 8 controls remained clinically normal throughout, no virus was isolated from their plasma and they did not develop any H.I. antibody to louping-ill virus.

Viraemia

At 24 hours after inoculation virus was isolated from plasma of all except 2 of those that were given virus. One susceptible animal (6) did not develop any detectable viraemia. The dynamics of viraemia in the 22 susceptible animals and 11 survivors that did circulate virus are summarized in Fig. 1. Maximum titres, ranging from 1×10^3 to 3×10^7, were recovered on days 3 and 4. The mean level of viraemia was slightly higher throughout in susceptible animals: in addition, maximum concentrations of virus recovered tended to be greater in those that succumbed, in 10 cases exceeding 10^6 p.f.u. per 0·2 ml. Titres of this magnitude were not detected in any of the survivors.

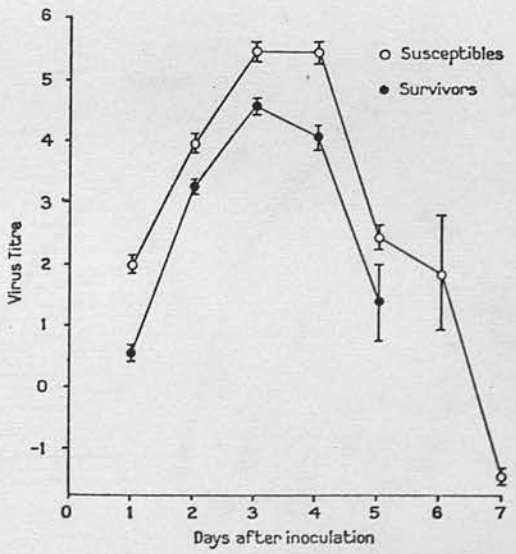
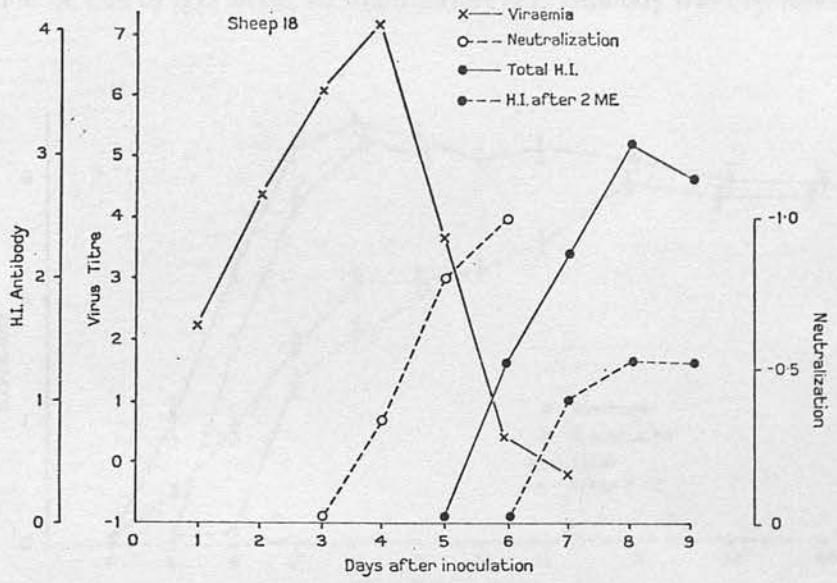
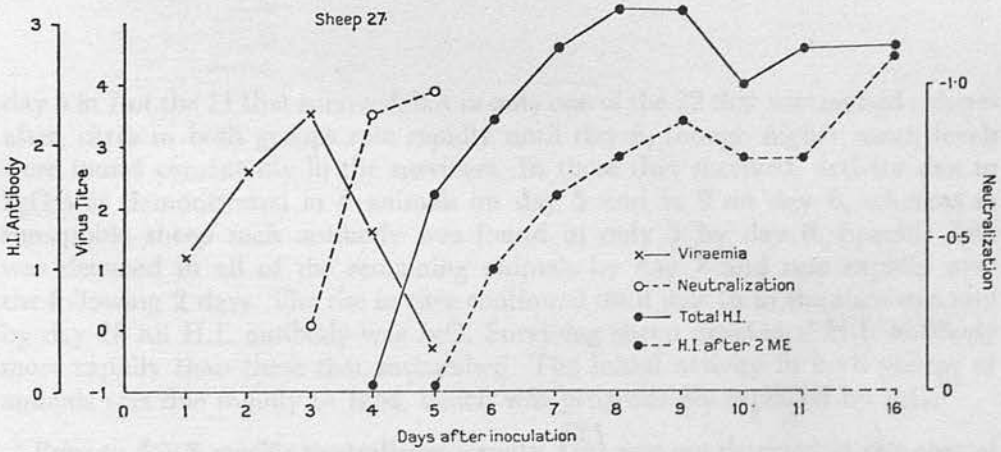


Fig. 1. Comparison of mean (± S.E.) daily levels of viraemia in 33 sheep inoculated s.c. with louping-ill virus. Twenty-two animals succumbed (susceptibles) and 11 survived. Virus titres are expressed as $\log_{10}$ p.f.u. per 0·2 ml. of plasma.

By day 4 titres had declined in 43 per cent. of the susceptible animals and 73 per cent. of the survivors. On day 5 levels of $>10^2$ p.f.u. per 0·2 ml. were still present in 13 of the susceptible animals, but were found in only 1 of the survivors. Viraemia persisted until days 6 and 7 in 81 per cent. and 33 per cent. respectively of the remaining susceptible sheep, but was not found subsequent to day 5 in any of those that survived. No animal was viraemic on the day of death, or when severe nervous symptoms developed. Thus, on average, levels





Figs 2 and 3. Patterns of development of viraemia, serum neutralizing capacity and serum H.I. antibodies in 2 typical cases. Sheep 18 succumbed at 9 days after inoculation and sheep 27 survived. Virus titres are expressed as $\log_{10}$ p.f.u. per 0·2 ml. of plasma. H.I. antibody titres are expressed as reciprocal of $\log_{10}$ titres in whole serum, or serum treated with 2ME (IgG).

of viraemia were higher in susceptible than in surviving sheep. Viraemia terminated earlier in those that survived.

Antibody Response

The decline in viraemia was associated with the appearance of circulating H.I. antibody and increasing levels of neutralizing activity in the serum (Figs. 2 and 3). The development of H.I. antibodies in susceptible and surviving sheep is summarized in Fig. 4. Antibody levels in whole serum represent activity in both the IgM and IgG classes, whereas titres remaining after treatment with 2ME are considered to be due to IgG alone. In whole serum H.I. antibody was first detected on

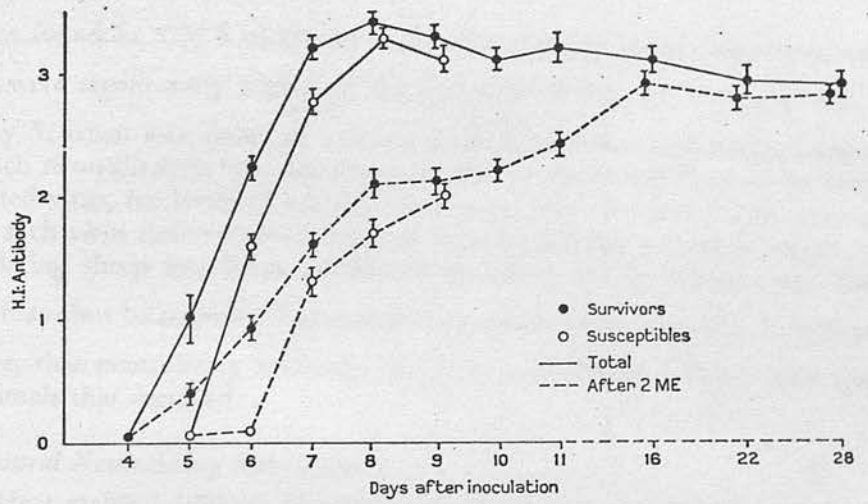


Fig. 4. The development of serum H.I. antibodies in susceptible and surviving sheep. Values expressed are mean (± S.E.) of $\log_{10}$ reciprocal titres in whole serum, or serum treated with 2ME (IgG).

day 5 in 7 of the 11 that survived, but in only one of the 22 that succumbed: thereafter, titres in both groups rose rapidly until day 8, though higher mean levels were found consistently in the survivors. In those that survived, activity due to IgG was demonstrated in 4 animals on day 5 and in 9 on day 6, whereas in susceptible sheep such antibody was found in only 3 by day 6. Specific IgG was detected in all of the remaining animals by day 7 and rose rapidly over the following 2 days. The rise in titre continued until day 16 in the survivors and by day 28 all H.I. antibody was IgG. Surviving sheep developed H.I. antibody more rapidly than those that succumbed. The initial activity in both groups of animals was due mainly to IgM, which was progressively replaced by IgG.

Prior to day 3 specific neutralizing activity $\left(\frac{S}{N}\right)$ was not detected in any animal given virus. Low levels were present in both susceptible and surviving groups by day 3 (Table 1). On day 4 sera from 10 of the 11 that survived specifically neutralized more than 50 per cent. of the virus inoculum, whereas such activity

TABLE 1

DEVELOPMENT OF NEUTRALIZING ACTIVITY* TO LOUPING-ILL VIRUS IN SERUM

Days after inoculation	*3*	*4*	*5*
Control sheep	$-0{\cdot}01 \pm 0{\cdot}01$	$+0{\cdot}10 \pm 0{\cdot}12$	$+0{\cdot}08 \pm 0{\cdot}05$
Surviving sheep	$-0{\cdot}12 \pm 0{\cdot}05$	$-0{\cdot}73 \pm 0{\cdot}04$	$-0{\cdot}94 \pm 0{\cdot}04$
Susceptible sheep	$-0{\cdot}11 \pm 0{\cdot}03$	$-0{\cdot}43 \pm 0{\cdot}06$	$-0{\cdot}90 \pm 0{\cdot}01$
Difference†	$P < 0{\cdot}4$	$P < 0{\cdot}0005$	$P < 0{\cdot}0125$

* Values of S/N are expressed as mean ± S.E.

† Difference between survivors and susceptibles, as calculated by Students t-test.

was found in only 8 of the 18 susceptible animals tested. The mean values for $\frac{S}{N}$ were significantly higher in the survivors (Table 1). This was also true on day 5, when sera from all animals showed high levels of neutralizing activity. Such neutralization was not obviously due to direct interference by heat inactivated virus, for levels of activity were in no way correlated with concentrations of such virus (inferred from titres of virus in plasma) present in serum. Any circulating sheep interferon would not be active in pig kidney cells. Values for $\frac{S}{N}$ may thus be considered as representing neutralizing antibody. It is clear, therefore, that neutralizing antibody, like H.I. antibody, developed more quickly in animals that survived.

Natural Neutralizing Substances

Heat stable inhibitors of virus were present in pre-inoculation sera from all except 3 of the 41 animals. The highest level of activity was found in sheep 6, which succumbed at 7 days after inoculation and was the only animal given virus that did not develop detectable viraemia. This serum reduced the plaque count by 86 per cent., leaving a residual fraction (N) of 0·14. Values for N in other animals ranged from 0·91 to 0·49. The mean (± S.E.) levels of N were $0{\cdot}62 \pm 0{\cdot}09$ in controls, $0{\cdot}69 \pm 0{\cdot}05$ in survivors and $0{\cdot}73 \pm 0{\cdot}04$ in those that succumbed. These values were not significantly different.

DISCUSSION

Virus was detected in plasma at 24 hours after inoculation in 90 per cent. of cases. This may reflect extensive early multiplication of louping-ill virus. The lag phase before production of new virus in sheep kidney cells in vitro is only 4 to 6 hours (Dr. J. T. Vantsis, personal communication). Primary multiplication in vivo probably occurs in the regional lymph nodes (Malkova, 1968), though small amounts of the inoculum may pass directly by the lymphatics to the blood (Malkova, Mayer and Vrubel, 1969). Any residual circulating inoculum would either be cleared quickly by reticulo-endothelial macrophages (Mims, 1964) or inactivated, for under in vitro conditions virus in suspension does not remain

viable for long at 37°C. (Czarkowska-Gladney and Hurst, 1931; unpublished data). In general the magnitude of viraemia was directly related to the severity of subsequent symptoms, though several susceptible animals circulated only low levels of virus and viraemia was not detected in one such case. Similar relationships have been demonstrated in rodents inoculated with louping-ill virus and other group B arboviruses. However in these experiments further major variables, such as host age (Doherty, 1969), partial immunity or treatment with immunosuppressive drugs (Thind and Price, 1969) were also present.

The exponential phase of viraemia was of shorter duration and the decline was much more rapid in sheep that survived. This was very definitely associated with the earlier appearance of both neutralizing and H.I. antibody in the survivors. Though low levels of interferon are also present from the day after inoculation (Dr. J. T. Vantsis, personal communication) termination of viraemia would seem to be largely mediated by antibody, as is recognized in other generalized virus infections (Fenner, 1968). The immune response of the host seems to be essentially protective. A similar conclusion has been made from the results of immunosuppression studies of other arbovirus infections in both rodents and monkeys (reviewed by Nathanson and Cole, 1970).

As observed in our previous study of louping-ill in sheep (Reid and Doherty, 1971) initial H.I. activity was due to IgM, which was progressively replaced by IgG. This sequence has also been observed in guinea pigs (Bellanti, Russ, Holmes and Buescher, 1965), rabbits (Westaway, 1968) and calves (Sanderson, 1968) inoculated with other group B arboviruses. The first neutralizing antibody detected was probably also IgM. The low avidity of early IgM (Svehag, 1965) may explain why circulating virus and neutralizing antibody could be demonstrated concurrently for several days.

The presence of heat-stable pre-existing inhibitors in serum did not obviously influence the outcome of infection. The highest level of activity was detected in a susceptible animal. Although these substances may have a high degree of specificity for different viruses (McFerran, 1962; Pagano, Gilden and Sedwick, 1965; Zilka, Kawaklova, Vicari and Archetti, 1968) their presence does not indicate prior exposure of the animal to viral antigen (Koprowski, 1946). In reviewing their role Ginsberg (1960) concluded that, in general, they are probably of little significance in the phenomenon of non-specific resistance to virus infection.

The incidence of fatal disease (66 per cent.) in these sheep may have been influenced by the daily withdrawal of 30·0 ml. of blood. In our previous experiment with this virus (Reid and Doherty, 1971), where only 2 of 8 animals succumbed and others developed non-fatal neurological symptoms, the usual daily blood sample was 2·0 ml. This considerable blood loss may simulate the stress of natural infestation with *Ixodes ricinus* ticks, the natural vector of louping-ill virus (Macleod and Gordon, 1932), though it was not sufficient to cause obvious debility in any of the controls. Physiological stress was also implicated by Gordon, Brownlee, Wilson and Macleod (1962) in discussing the death of 33 of 50 sheep inoculated s.c. with a "vaccine" strain of virus which, in a preliminary experiment, had caused only mild febrile symptoms with no evidence of neurological involvement.

Under field conditions all animals with viraemia would have been of potential

epidemiological significance, because levels of virus detected in plasma were sufficient to infect *I. ricinus* ticks (Swanepoel, 1968; Radda, Hofman and Pretzmann, 1969).

SUMMARY

Of 33 six-month-old Scottish Blackface sheep inoculated subcutaneously with a high dose of louping-ill virus, 22 developed fatal neurological disease at between 7 and 12 days after inoculation and 11 survived without obvious symptoms of encephalomyelitis. Virus was demonstrated in plasma from 90 per cent. of these sheep at 24 hours after inoculation.

Circulating virus was detected in all except one animal, which was moribund on day 7. Both mean and maximum levels of viraemia tended to be higher in those that succumbed. Viraemia terminated much more rapidly in the survivors. This was very definitely associated with the earlier appearance of both neutralizing and haemagglutination inhibiting antibodies in sheep that survived. The immune response in louping-ill seems to be essentially protective. Initial activity was due to IgM, which was progressively replaced by IgG. Neutralizing antibody and virus circulated concurrently for several days. Sera from all except 3 animals contained pre-existing heat-stable neutralizing substances, which had no obvious influence on the outcome of infection. No symptoms were seen in any of 8 control sheep.

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LOUPING-ILL ENCEPHALOMYELITIS IN THE SHEEP

II. Distribution of Virus and Lesions in Nervous Tissue

By

P. C. Doherty and H. W. Reid

Moredun Research Institute, Gilmerton, Edinburgh

INTRODUCTION

In sheep with louping-ill pathological changes are unevenly distributed throughout the central nervous system (C.N.S.). The most severe lesions are seen in the grey matter of the brainstem, cerebellum and spinal cord, whereas the cerebrum is relatively undamaged (Doherty and Reid, 1971). As this pattern may reflect a differential susceptibility of cells in nervous tissue to infection with louping-ill virus, the distribution of pathological changes and virus in the C.N.S. should correspond. This relationship is examined in the present paper.

MATERIALS AND METHODS

The subcutaneous (s.c.) inoculation of 33 sheep with a high dose of louping-ill virus and 8 sheep with control material was described in the previous paper (Reid and Doherty, 1971).

Autopsy. Cerebrospinal fluid (C.S.F.) was withdrawn from the cisterna magna immediately prior to death. Sheep with louping-ill were killed when moribund. Small pieces of nervous tissue were taken aseptically from the dorsum of the right cerebral hemisphere, the posterior aspect of the cerebellum and the anterior cervical spinal cord. These samples, and the C.S.F., were stored at −70°C. for virus titration studies.

The brain was divided sagitally. One half was taken into formol saline. The other half was cut into 5·0 mm. coronal slices, some of which were placed in small polythene bags. These were sealed, quenched in an eutectic mixture (dry ice and petroleum ether, −70°C.) and stored at −20°C. Blocks of cervical spinal cord were treated in the same way.

Material was also examined from 6 controls and 4 surviving animals killed at one month after inoculation.

Virus titration. Samples of nervous tissue were thawed, homogenized as 10 per cent. suspensions in tissue culture growth medium and centrifuged at 1500 *g* for 15 minutes at 4°C. Concentrations of virus in the supernates and in thawed samples of C.S.F., were determined using the plaque assay system described previously (Reid and Doherty, 1971). Virus titres were expressed as p.f.u. per 0·2 g. of tissue, or per 0·2 ml. of C.S.F.

Immunofluorescence. A serum with a very high haemagglutination inhibiting titre (1:250,000) to louping-ill virus was produced by hyperimmunizing a sheep with a methanol precipitated adjuvant vaccine (Brotherston and Boyce, 1970). The globulins were fractionated by ammonium sulphate precipitation and conjugated with fluorescein isothiocyanate, using the dialysis method of Clarke and Shepard (1963). This conjugated globulin, diluted 1:1 in phosphate buffered saline (P.B.S.) and

absorbed once with mouse liver powder and once with sheep liver powder (Nairn, 1962), was then centrifuged at 18,000 *g* for 30 mins. at 4°C., filtered through a millipore membrane and stored in ampoules at −70°C. These were thawed as required and the contents were further diluted 1:7 in P.B.S.

Blocks of frozen nervous tissue were trimmed with a cold scalpel blade and mounted, with 2 drops of 15 per cent. ovalbumin, on cryostat chucks. Sections (8 μ), cut at −18°C. and lifted off the knife with cleaned glass slides, were fan-dried for 10 mins., fixed in cold acetone (+4°C.) for 10 mins. and dried again for a further 10 mins. They were then overlaid with the diluted conjugate and incubated in a humid chamber for 30 mins. at 37°C. This was followed by 3 ten-minute washings in P.B.S. and one 5-minute washing in distilled water. Sections were then mounted in glycerol buffered to pH 8·0 and examined in a fluorescence microscope.

The specificity of this anti-louping-ill conjugate was established both by staining control sections from normal animals and by inhibiting fluorescence using a blocking test (Nairn, 1962). Sections from positive cases were first overlaid with un-conjugated specific immune serum. After 30 mins. this was washed off, and the sections were stained with the conjugate and washed again. Greater than 80 per cent. inhibition of fluorescence was observed in every case. The number of cells showing strong apple-green fluorescence was estimated subjectively and graded from + to + + + +. At least 3 sections, on 3 separate slides, were examined at random from each block. These 3 readings were averaged to give the values quoted in the results.

Pathology. The half brains that were fixed in formol saline were processed as described previously (Doherty and Reid, 1971). Coronal blocks were embedded in paraffin and 6 μ sections were stained with haematoxylin and eosin (H. & E.). The severity of neuropathological changes was graded 1 to 4 by the criteria of Nathanson, Goldblatt, Thind, Davis and Price (1965).

RESULTS

The virus was isolated from nervous tissue of all moribund animals (Table 1), but not from any of 6 controls or 4 survivors killed one month after inoculation. The highest titres recovered were from the spinal cord (88 per cent. of cases) and from the cerebellum of the remainder. Lower levels of virus were isolated less consistently from the cerebrum. Virus was detected in only 1 of the 12 C.S.F. samples examined. The isolation technique was more sensitive than immunofluorescence for detecting virus in the C.N.S. Concentrations of approximately 1,000 p.f.u. per 0·2 g. of tissue were necessary before a positive result was recorded by the fluorescent test (Tables 1 and 2). Even so fluorescing cells were observed in all cases.

Specific fluoresence could only be identified in nerve cells, and no evidence of infection of glia or vascular endothelium was found. The apple-green staining was mainly confined to neuronal cytoplasm, often in a rather "sponge-like" pattern (Figs. 1 and 2). Fluorescence tended to fill the cytoplasm and sometimes extended into neuronal processes (Figs. 3, 4 and 5), though not for any great distance, as none was seen in the long tracts of the spinal cord. Less intense staining was seen in many cells (Figs. 5 and 6), probably because they were severely damaged.

The distribution of fluorescence in cryostat sections from one half of the brain corresponded fairly well to the pattern of lesions in paraffin sections from the other half (Table 2). Eighty-six per cent. of maximum readings for immunofluorescence were recorded from the brainstem and spinal cord, as were 95 per

TABLE 1

CONCENTRATIONS OF VIRUS* IN NERVOUS TISSUE OF SHEEP WITH LOUPING-ILL

Animal No.	*Day*	*Cerebrum*	*Cerebellum*	*Spinal cord*	*C.S.F.*
1	6	$3{\cdot}4 \times 10^3$	$7{\cdot}9 \times 10^4$	$2{\cdot}7 \times 10^3$	NE
2	7	$1{\cdot}7 \times 10^5$	$1{\cdot}7 \times 10^7$	NE	NE
3	7	$3{\cdot}4 \times 10^1$	$8{\cdot}6 \times 10^2$	$1{\cdot}6 \times 10^4$	0
4	7	$1{\cdot}7 \times 10^4$	$3{\cdot}6 \times 10^5$	$2{\cdot}6 \times 10^5$	0
5	7	$7{\cdot}6 \times 10^3$	$1{\cdot}5 \times 10^4$	$3{\cdot}1 \times 10^4$	0
6	7	$8{\cdot}0 \times 10^3$	$1{\cdot}2 \times 10^4$	$3{\cdot}4 \times 10^4$	NE
7	8	$1{\cdot}2 \times 10^4$	$4{\cdot}0 \times 10^5$	$3{\cdot}2 \times 10^6$	$3{\cdot}9 \times 10^1$
8	8	$5{\cdot}8 \times 10^3$	$5{\cdot}3 \times 10^4$	$3{\cdot}0 \times 10^5$	NE
9	8	$4{\cdot}2 \times 10^3$	$2{\cdot}6 \times 10^4$	$1{\cdot}0 \times 10^6$	0
10	8	0	$3{\cdot}6 \times 10^2$	$2{\cdot}8 \times 10^5$	NE
11	8	$2{\cdot}8 \times 10^2$	$1{\cdot}1 \times 10^4$	$6{\cdot}5 \times 10^5$	0
12	8	$1{\cdot}6 \times 10^3$	$2{\cdot}3 \times 10^4$	$5{\cdot}8 \times 10^5$	NE
13	8	$1{\cdot}9 \times 10^4$	$2{\cdot}4 \times 10^6$	$2{\cdot}3 \times 10^6$	0
14	9	$1{\cdot}5 \times 10^2$	$6{\cdot}3 \times 10^4$	$6{\cdot}3 \times 10^5$	NE
15	9	0	$2{\cdot}6 \times 10^2$	$1{\cdot}1 \times 10^5$	NE
16	9	$1{\cdot}6 \times 10^3$	$3{\cdot}6 \times 10^5$	$4{\cdot}2 \times 10^5$	NE
17	9	0	$2{\cdot}3 \times 10^2$	$3{\cdot}4 \times 10^3$	0
18	9	NE	$4{\cdot}6 \times 10^3$	$1{\cdot}6 \times 10^5$	0
19	9	$3{\cdot}8 \times 10^3$	$1{\cdot}5 \times 10^4$	$3{\cdot}0 \times 10^5$	NE
20	9	$2{\cdot}2 \times 10^3$	$2{\cdot}4 \times 10^3$	$4{\cdot}4 \times 10^5$	0
21	10	$0{\cdot}2 \times 10^1$	$1{\cdot}2 \times 10^2$	$1{\cdot}6 \times 10^3$	0
22	12	0	NE	$6{\cdot}0 \times 10^3$	0

* = Mean virus titre, expressed as p.f.u. per 0·2 g. of nervous tissue.
0 = No virus isolated.
NE = Not examined.

cent. of the most severe pathological changes observed. The cerebellum was less consistently affected, though high concentrations of viral antigen and marked lesions were detected in a minority of cases. Inflammatory changes were present in the cerebral cortex of most moribund animals, but both fluorescent and necrotic neurons were relatively uncommon. No fluorescence was seen in nervous tissue from any of the 4 survivors examined. Slight lesions, given a maximum grade of 1, were found in each case.

DISCUSSION

The distributions of both infectious virus and viral antigen correspond to the pattern of damage to nerve cells. Neuron necrosis would thus seem to represent the direct cytopathic effect of virus growth within the cell. Louping-ill virus is known to cause rapid lysis of unprotected populations of susceptible cells (Burnet, 1936; Williams, 1958). The development of symptoms of louping-ill may be accounted for by the extensive neuronal loss resulting from virus replication. Extrinsic factors, such as functional load and oedema (Webb, 1968), may also play an ancillary role in the development of neurological dysfunction.

Viral antigen was observed only in neuronal cytoplasm. El Dadah and Nathanson (1967) were also unable to identify virus in other than nerve cells in the brains of rats injected with the West Nile group B arbovirus. In ultrastructural

TABLE 2

DISTRIBUTION OF FLUORESCENT NEURONS AND PATHOLOGICAL CHANGES IN SHEEP WITH LOUPING-ILL

		*Fluorescent neurons**					*Pathological changes**				
			Cerebellar cortex					*Cerebellar cortex*			
Animal number	*Days after inoculation*	*Cerebral cortex*	*Purkinje*	*Granular*	*Pons*	*Ventral horn*	*Cerebral cortex*	*Purkinje*	*Granular*	*Pons*	*Ventral horn*
1†	6	−	−	−	+	−	0	0	0	0	0
2	7	++	+++	+	++++	++++	1	3	2	4	3
3	7	−	−	−	−	+	1	3	2	3	2
4	7	+	+++	++	++	++	1	3	2	4	4
5	7	−	++++	+++	+++	++	1	2	1	2	2
6	7	−	+	−	++++	++	1	2	1	2	2
7	8	−	+	+	++++	+++	2	1	1	2	3
8	8	+	+	+	NE	NE	1	2	2	3	3
9	8	−	+	−	+++	NE	1	3	2	3	3
10	8	−	−	−	++	++	1	3	2	3	2
11	8	−	+	−	+++	+++	1	2	2	3	2
12	8	−	++	++	+++	+++	1	2	2	4	3
13	8	−	+	−	+++	NE	1	1	1	2	2
14	9	−	++++	++	++	++++	1	2	1	4	4
15	9	−	−	−	++	++	1	1	1	2	1
16	9	−	+	−	++	++++	1	0	0	2	3
17	9	−	−	−	+	++	1	1	1	2	2
18	9	+	−	−	++	+++	1	1	1	2	3
19	9	++	++	++	++	++++	1	2	2	3	3
20†	9	−	−	−	++	++++	0	1	1	1	2
21†	10	NE	+	−	+++	NE	1	1	1	2	2
22	12	−	−	−	−	+	0	1	1	1	2

* = The number of fluorescent neurons was graded from + to ++++, the severity of histological changes from 1 to 4.
† = These animals died, the remainder were killed in extremis.
− = No fluorescence detected. 0 = No lesions detected NE = Not examined.

studies of rodent nervous tissue particles of louping-ill virus were also localized in neuronal cytoplasm, usually enclosed in abnormal membrane profiles (Smith and Doherty, 1969; Zlotnik and Harris, 1970). Other groups B arbovirus have been detected, however, by immunofluorescence, in both glial cells and the endothelium of mouse brain (reviewed by Johnson and Mims, 1968).

The distribution of virus and lesions is essentially similar to that observed in monkeys inoculated with other viruses of the tick-borne encephalitis complex (Nathanson, Gittelsohn, Thind and Price, 1967; Mayer and Rajcani, 1968). There are well recognised functional relationships (Truex and Carpenter, 1964) between regions of the C.N.S. that were severely damaged. It is unlikely, however, that the pattern of nerve cell necrosis results from spread of virus along preferential neuronal pathways, as was suggested for cats infected with Newcastle disease virus by Luttrell and Bang (1958). Though louping-ill virus may be detected in axons and dendrites, it was not found at any great distance from the cell body.

The Purkinje cells were much less consistently damaged in these s.c. inoculated sheep than they were in animals given virus intracerebrally (Doherty and Reid, 1971). The cerebellar lesion in louping-ill has been somewhat over-emphasised, probably because most descriptions are of animals injected by the intracerebral route (Hurst, 1931; Brownlee and Wilson, 1932). The Purkinje cells of natural cases are often relatively unaffected (unpublished data).

The high concentrations of antibody found in C.S.F. (Reid, Doherty and Dawson, 1971) probably neutralised any free virus that was present. Even so, virus was recovered from nervous tissue in every case. This was not due to concurrent presence of viraemic blood, as virus was not isolated from plasma of any moribund animal (Reid and Doherty, 1971). Maximum levels of virus were found most consistently in the spinal cord. Examination of spinal cord for the presence of virus may be of value in the diagnosis of naturally occurring cases.

SUMMARY

Louping-ill virus was demonstrated consistently, by both virus isolation and immunofluorescence, in nervous tissue from moribund sheep. In only one case was virus detected in cerebrospinal fluid. Viral antigen was observed only in neuronal cytoplasm. The distributions of virus and nerve cell damage corresponded. The highest concentrations of virus were found in the brainstem and spinal cord, where neuron necrosis was also most apparent. The cerebellum was less uniformly affected. Lesions in the cerebrum were predominantly inflammatory, and virus was detected less frequently and in smaller amounts. There were slight pathological changes in surviving animals, but virus could not be demonstrated in nervous tissue.

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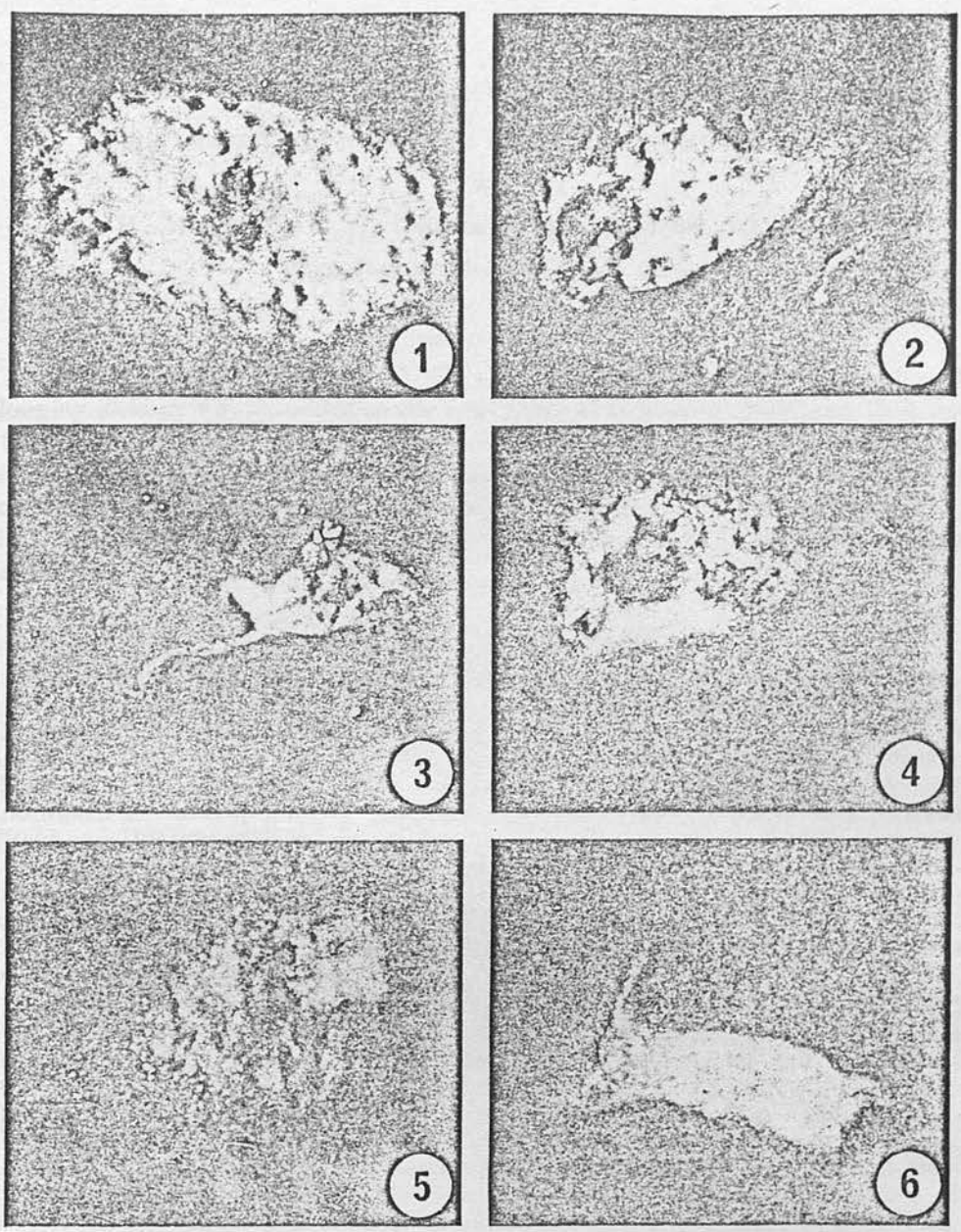

Figs 1 to 6. Fluorescent neurons in the pons of sheep 5, which was moribund at 7 days after s.c. inoculation with virus. The absence of antigen from the nucleus is apparent in Figs. 1, 2, 4 and 5. Fluorescence is seen in neuronal processes in Figs. 3, 4 and 6. The less intense staining shown in Figs. 5 and 6 may represent a late stage of infection of the cell. Fluorescent antibody × 720.

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LOUPING-ILL ENCEPHALOMYELITIS IN THE SHEEP

III. Immunoglobulins in Cerebrospinal Fluid

By

H. W. Reid, P. C. Doherty and A. McL. Dawson

Moredun Research Institute, Gilmerton, Edinburgh

INTRODUCTION

The serum antibody response of sheep inoculated subcutaneously (s.c.) with louping-ill virus was described in the first paper of this series (Reid and Doherty, 1971b). Detailed studies of immunoglobulins in terminal serum and cerebrospinal fluid (C.S.F.) samples from some of these animals are recorded in the present communication. The aim was to determine whether specific antibody is present in C.S.F. from sheep with louping-ill and to relate any such activity to circulating antibody.

MATERIALS AND METHODS

Experimental. The 41 sheep used in these experiments were each injected intramuscularly with 1·0 ml. of a 20 per cent. w/v solution of clarified egg albumin.* The intention was to provide a circulating antibody which was in no way involved in the infectious process and could thus be used as a serum marker (Webb, Connolly, Kane, O'Reilly and Simpson, 1968). Twenty-two days later 33 of these animals were inoculated s.c. with louping-ill virus, and the other 8 were given control material (Reid and Doherty, 1971b).

Terminal blood samples were taken from the jugular vein and C.S.F. was withdrawn from the cisterna magna. Only C.S.F. samples that were free of obvious contamination with blood were examined further. The quantities of C.S.F. collected ranged from <1·0 ml to 5·0 ml. These volumes were insufficient to allow all tests to be performed on each case.

Antibody titration. The passive haemagglutination (P.H.A.) test (Herbert, 1967) was used to measure antibody to egg albumin in both serum and C.S.F. Serum haemagglutination inhibiting (H.I.) antibody titres to louping-ill virus were determined as described previously (Reid and Doherty, 1971a). In control C.S.F. non-specific inhibitors were detected only at a dilution of 1:3 or less. Therefore H.I. titres in C.S.F. were assessed without prior extraction, as is necessary for serum. Levels of H.I. antibody were also measured after treating serum with 0·2M 2-Mercaptoethanol (2ME), which inactivates IgM but not IgG. As smaller amounts of protein were present in C.S.F., 2ME was used at a lower final concentration (0·01M).

The relative H.I. activities in IgG and IgM classes of antibody were also assessed by Sephadex G-200 filtration of paired serum and C.S.F. samples. Two ml. aliquots of serum were extracted with acetone, as described by Clarke and Casals (1958) except that the final acetone precipitate was reconstituted in 2·0 ml. of tris-HCl buffer (0·1M tris hydroxymethyl-methylamine + 1·0M sodium chloride, pH 8·0). One ml. vols. of extracted serum, or unextracted C.S.F., were applied to a column (2·5 × 57 cm.) which had been equilibrated with tris-HCl buffer. The first 2 exclusion peaks were collected and the H.I. activity of the fractions was determined.

* B.D.H., Poole, Dorset.

Quantitation of Immunoglobulins

The total levels of IgG and IgM in serum and C.S.F. were measured by the single radial diffusion technique of Mancini, Carbonara and Heremans (1965), as modified by Fahey and McKelvey (1965). The specific antisera necessary for this method were prepared by inoculating rabbits with purified IgG and IgM isolated from pooled normal sheep serum.

Preparation of purified IgG. Thirty ml. of sheep serum was dialysed against 0·02M phosphate buffer (PO_4) at pH 7·4, and then filtered through Whatman DE32 ion exchange cellulose (column size 1·5 × 25 cm.) which had been equilibrated with the same buffer. The fall-through eluate was collected and concentrated to 7·0 ml. by force dialysis against tris-HCl buffer. Two 3·5 ml. vols. of this concentrate were filtered through a column (2·5 × 73 cm.) packed with Sephadex G-200 which had been equilibrated with the same buffer. The central portion of the major peak was concentrated and recycled through Sephadex G-200. A single symmetrical peak emerged, which was concentrated by force dialysis against phosphate buffered saline (P.B.S., pH 7·4) to a final volume of 8·0 ml. This was lyopholised and stored at −70°C. On Kjeldahl analysis this material was found to contain 23·44 mg. of protein per ml. (N × 6·25). A single precipitin line appeared on immunoelectrophoresis, which corresponded in position and appearance to IgG when reacted against rabbit anti-whole sheep serum.

Preparation of purified IgM. Two litres of serum, diluted 1:20 in distilled water to precipitate the euglobulins, was kept overnight at +4°C., and then centrifuged at 1800 *g* for 60 minutes at 4°C. The resulting precipitate was dissolved in tris-HCl buffer, to give a final volume of approximately 11·0 ml., and clarified by centrifuging at 1800 *g* for 20 minutes. One ml. aliquots of the supernate were filtered through a Sephadex G-200 column (2·5 × 57 cm.), which had been equilibrated with the same buffer, and the first exclusion peak from each filtration was collected. The first half of these peaks was concentrated by force dialysis against 0·075M PO_4 (pH 8·0). This concentrate was applied to a D.E.A.E. Sephadex A50 (particle size 40–120 μ) column (1·5 × 25 cm.), which had been equilibrated with the same buffer, and 50 ml. of buffer was run through. The column was then eluted with 50 ml. of 0·125M PO_4 followed by 50 ml. of 0·20M PO_4. A major peak, which eluted with this latter concentration of PO_4, was force dialysed against P.B.S. (pH 7·4) to 4·0 ml. and stored at −70°C. in sealed ampoules. The final concentrate contained 2·58 mg. of protein per ml. On immunoelectrophoresis a single precipitin band, which was identical to that of IgM, was produced.

Preparation of antisera to IgG and IgM. The purified immunoglobulins were emulsified with Freund's incomplete adjuvant and 1·0 ml. aliquots were injected into mature New Zealand white rabbits by both the s.c. and i.v. routes. These rabbits received further injections, without adjuvant, one month later. They were bled after a further interval of 10 days, and periodically thereafter. Booster injections were given as required. The specificity of the antisera produced was assessed by immunoelectrophoresis (Scheidegger, 1955) and agar double diffusion. Antisera to IgG formed only one precipitin line when reacted against whole sheep serum: however antisera to IgM also reacted weakly against IgG. Antisera to IgM were, therefore, absorbed with approximately 5·0 mg. of IgG per ml., at 37°C. for 2 hours and overnight at +4°C. After this procedure only one precipitin line, which was typical of IgM in both position and appearance, was present when reacted with whole sheep serum.

Single radial diffusion technique. Wells were cut 1·25 cm. apart in agar layered on 10 × 8 cm. photographic plates. Dilutions of standard immunoglobulins, together with the test samples, were added to each plate and were allowed to react at room temperature for 24 hours with IgG determinations, and 48 hours with IgM determinations. The plates were then immersed in isotonic saline for 48 hours to stop the reaction, dried and stained with 0·1 per cent. Azocarmine B in 5 per cent. acetic

acid. The stained ring diameters were measured using a photographic enlarger. The ring diameters of both the IgG and IgM precipitates were found to be directly related to the $\log_{10}$ concentration of protein. Test samples of serum were diluted so that the ring diameters were of the same order as found in undiluted C.S.F. Hence for IgG determinations serum was diluted 200 times and for IgM determinations 10 and 20 times.

RESULTS

Three groups of sheep are considered: those moribund following s.c. inoculation with virus, those surviving such exposure and the controls.

High levels of H.I. antibody were present in serum and C.S.F. from all moribund and surviving sheep that were studied (Table 1). No H.I. activity was found in control serum, though there was a low level of non-specific activity in control C.S.F. Antibody (P.H.A.) to egg albumin was detected in serum and C.S.F. from all animals, at approximately equivalent titres in each group. The levels of P.H.A. antibody in C.S.F. were generally very low. In all 3 groups the concentrations of P.H.A. antibody in serum were approximately 160 times greater than those in C.S.F. On average, however, the level of H.I. antibody in serum from sheep given virus was only 10 or 20 times greater than that in C.S.F. Thus,

TABLE 1

LEVELS* OF H.I. ANTIBODY TO LOUPING-ILL VIRUS AND P.H.A. ANTIBODY TO EGG ALBUMIN IN SERUM AND C.S.F.

Animal	*Days after inoculation*	*H.I.†*			*P.H.A.*		
		Serum	*C.S.F.*	*Ratio*	*Serum*	*C.S.F.*	*Ratio*
4	7	>1280	>64	20	2560	<2	1280
5	7	640	128	5	320	4	80
11	8	5120	512	10	320	4	80
17	9	>1280	>128	10	5120	32	160
20	9	1280	128	10	2560	8	320
22	9	1280	32	40	640	2	320
24	30	1280	>64	<20	1280	2	640
28	77	>320	16	20	160	<2	160
29	78	640	32	20	80	<2	80
30	Control	0	2		320	2	160
31	„	0	<2		160	<2	160
32	„	0	2		320	4	80

* Antibody titres are expressed as reciprocals.
† The serum samples were extracted (Clarke and Casals, 1958) prior to testing. The C.S.F. samples were not extracted.

relative to the titres in serum, there was considerably more specific antibody to louping-ill virus in C.S.F. from moribund and surviving sheep than there was to an antigen not involved in the infectious process. When samples from moribund animals were treated with 2ME the levels of H.I. activity were considerably reduced in every case (Table 2). The degree of reduction in titre was comparable in serum and C.S.F. In surviving animals, however, levels of H.I. activity were unaffected by exposure to 2ME. By this criterion most H.I. antibody in serum and C.S.F. from clinical cases is IgM, whereas all H.I. antibody in surviving animals is IgG.

TABLE 2

EFFECT OF TREATMENT WITH 2ME ON LEVELS* OF H.I. ANTIBODY IN SERUM AND C.S.F.

Animal	Days after inoculation	Serum			C.S.F.		
		P.B.S.†	2ME	Ratio	P.B.S.†	2ME	Ratio
4	7	>1280	20	64	48	<3	>16
9	8	5120	>160	32	192	3	64
11	8	5120	>80	64	384	6	64
15	9	>2560	>80	32	384	24	16
17	9	1280	160	8	192	3	64
18	9	>640	10	64	96	6	16
20	9	>640	>80	8	96	6	16
21	10	2560	320	8	192	6	32
22	12	>1280	>320	4	48	12	4
23	28	>1280	>1280	1	96	96	1
24	30	1280	1280	1	48	48	1
25	30	>1280	>1280	1	192	192	1
26	35	>320	640	<1	48	48	1
27	35	>320	>320	1	192	192	1
28	77	>320	>320	1	48	48	1

* Antibody titres are expressed as reciprocals.
† Samples were treated with P.B.S. as a control.

These findings were confirmed when samples of serum and C.S.F. from 6 moribund sheep, 2 survivors and 1 control were filtered through Sephadex G-200 and titres of H.I. antibody in the eluates were determined. There was insufficient protein to cause any U.V. absorption in the eluates of C.S.F., but the position of the exclusion peaks could be inferred from the results with serum. In serum and C.S.F. from acute cases, e.g. Fig. 1, most H.I. antibody was present in the first exclusion peak (IgM) whereas in survivors, e.g. Fig. 2, all activity was detected in the second exclusion peak (IgG). No H.I. activity was found in eluates from control serum or C.S.F.

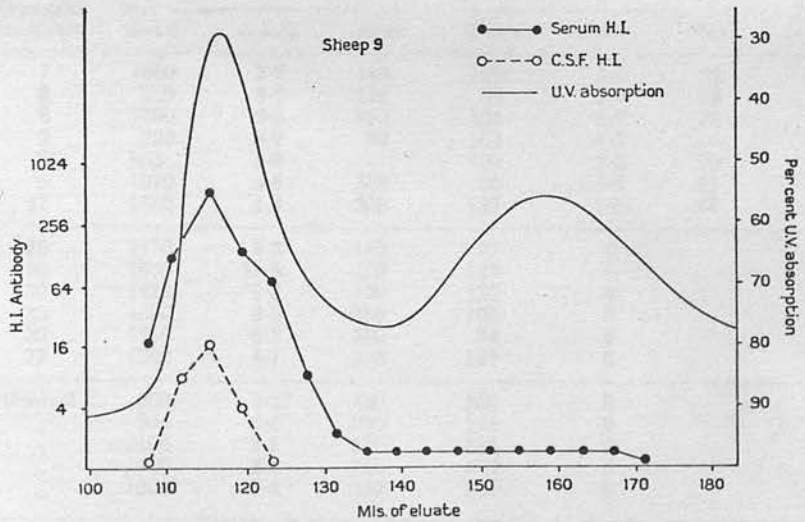

Fig. 1. Distribution of H.I. activity following Sephadex G-200 filtration of serum and C.S.F. from sheep 9, which was moribund at 8 days after inoculation with virus.

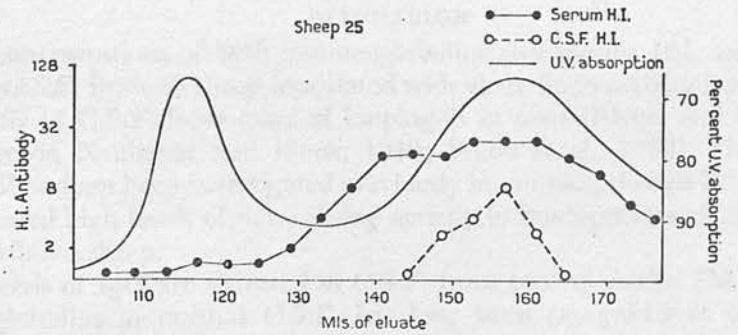

Fig. 2. Distribution of H.I. activity following Sephadex G-200 filtration of serum and C.S.F. from sheep 25, which was chronically debilitated and was killed at 30 days after inoculation with virus.

These results were further supported by the observation that there was no detectable IgM in C.S.F. from control or surviving sheep, though quite large amounts were present in C.S.F. from acute cases (Table 3). Control C.S.F. contains some IgG, but at significantly lower levels than are found in moribund ($p < 0.025$) or surviving ($p < 0.01$) animals. Levels of immunoglobulins in serum were not significantly different for the 3 groups. Protein levels in C.S.F. from acute cases were also significantly greater ($p < 0.0025$) than those in controls, but the difference between controls and survivors was not significant.

TABLE 3

TOTAL IMMUNOGLOBULINS* IN SERUM AND C.S.F. AND PROTEINS IN C.S.F.

Animal	*Days after inoculation*	*IgG*			*IgM*			*Protein in C.S.F.*
		Serum	*C.S.F.*	*Ratio*	*Serum*	*C.S.F.*	*Ratio*	
4	7	1060	7·7	140	109	5·1	21	111·9
9	8	950	3·5	270	89	3·7	24	37·7
11	8	1260	3·8	330	101	4·7	22	ND
17	9	660	8·2	80	ND	4·7	—	76·9
18	9	ND	5·0	—	109	3·6	30	51·7
20	9	1070	3·6	300	96	4·5	21	71·3
22	12	1660	4·4	380	137	3·4	40	48·5
23	28	1150	8·3	140	77	0		46·7
24	30	1600	13·5	120	120	0		72·8
25	30	1420	9·2	150	130	0		47·0
26	35	1500	4·3	350	105	0		19·5
27	35	1300	6·7	200	94	0		35·0
28	77	1260	4·7	270	121	0		22·1
33	Control	950	2·17	430	108	0		12·5
34	,,	950	3·8	250	105	0		38·1
35	,,	2160	3·6	270	128	0		19·0
36	,,	980	3·5	260	ND	0		22·9
37	,,	1020	2·8	370	153	0		17·4

* Expressed as mg. per 100 ml.
ND = Not done. 0 = No immunoglobulin detected.

DISCUSSION

High concentrations of both immunoglobulins and specific H.I. antibodies are found in C.S.F. from all sheep inoculated with virus. Such activity is also detected consistently in C.S.F. from cases of louping-ill in man (Rivers and Schwentker, 1934; Brewis, Neubauer and Hurst, 1949; Webb *et al.*, 1968). This aspect of louping-ill had not been investigated previously in animals, though Edward (1949) demonstrated high levels of neutralizing activity in homogenates of nervous tissue from moribund sheep.

Low levels of IgG are detected in C.S.F. from control sheep. The presence of immunoglobulins in normal C.S.F. has long been recognised in other species (Freund, 1930). According to Heremans (1968) such activity is always confined to IgG class antibody, which originates by filtration from plasma through a "sieve" of sufficiently small pore size to exclude IgM. The low C.S.F. titres to egg albumin may be largely non-specific, or may be explained by this mechanism. The serum/C.S.F. ratios for P.H.A. antibody in sheep are of a similar order to those recognised in normal rabbits inoculated with the same antigen (Sherwin, Richter, Cosgrove and Rose, 1963).

The proportion of P.H.A. antibody detected in C.S.F. was not obviously increased in either moribund or surviving sheep. Thus, it may be inferred that the rate of filtration of circulating immunoglobulins into the C.S.F. was not significantly affected by the infectious process. Concentrations of protein were, however, very much higher in C.S.F. from acute cases. These increased levels may have originated from lysed inflammatory cells, as protein determinations were made on C.S.F. that had been frozen without prior centrifugation. Elevated white cell counts have been demonstrated in C.S.F. from men (Rivers and Schwentker, 1934; Brewis *et al.*, 1949; Webb *et al.*, 1968), monkeys (Galloway and Perdrau, 1935) and cattle (Dunn, 1952) with louping-ill.

The relative proportions of IgM and IgG specific to louping-ill virus are similar in serum and C.S.F. In clinical cases most globulin in C.S.F. is IgM. The molecular weight of IgM is about 6 times that of IgG (Humphrey and White, 1964). Any alteration in permeability of the choroid plexuses or vascular walls would tend to favour escape of the smaller IgG molecule. The pattern of immunoglobulins in C.S.F. cannot, therefore, be explained on a basis of increased filtration of circulating antibody. The high levels of specific antibody to louping-ill virus that are detected in C.S.F. must, by default, originate locally in the central nervous system. A similar conclusion concerning the source of specific antibody in nervous tissue or C.S.F. has been made from studies of poliomyelitis (Morgan, 1947), rabies (Bell, Lodmell, Moore and Ramond, 1966), subacute sclerosing panencephalitis (Cutler, Merler and Hammerstad, 1968; Ter Meulen, Enders-Ruckle, Muller and Joppich, 1968) and distemper (Cutler and Averill, 1969).

In serum and C.S.F. from acute cases H.I. activity is associated with both IgM and IgG whereas in survivors, or sheep vaccinated some time previously (unpublished data), all such antibody is IgG. The clinical disease can thus be readily differentiated from other neurological conditions, where pre-existing antibody to louping-ill virus may occur incidentally. A definitive diagnosis can be made by determining levels of H.I. antibody in terminal serum and C.S.F., before and after treatment with 2ME.

SUMMARY

Specific haemagglutination inhibiting antibody is detected invariably in cerebrospinal fluid from sheep that have been inoculated subcutaneously with louping-ill virus. Much of the antibody in serum and cerebrospinal fluid from acute cases is IgM, whereas in survivors all specific activity is due to IgG. This pattern is reflected in the total concentrations of these two classes of immunoglobulins in cerebrospinal fluid. The relative proportion of haemagglutination inhibiting antibody detected in cerebrospinal fluid was considerably greater than that for a marker antibody, to an antigen not involved in the infectious process. Antibody specific to louping-ill virus is thus apparently produced locally in the central nervous system.

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